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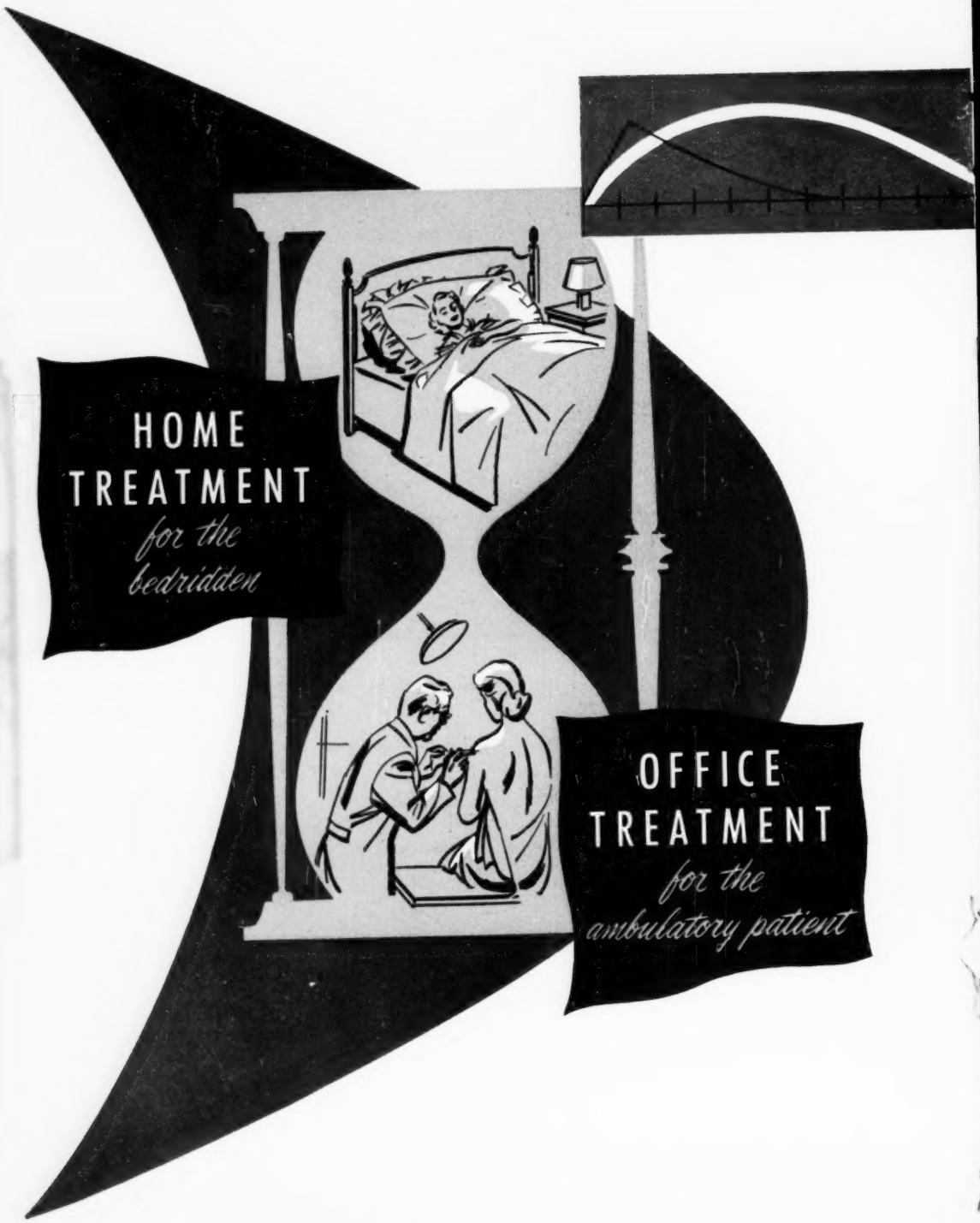
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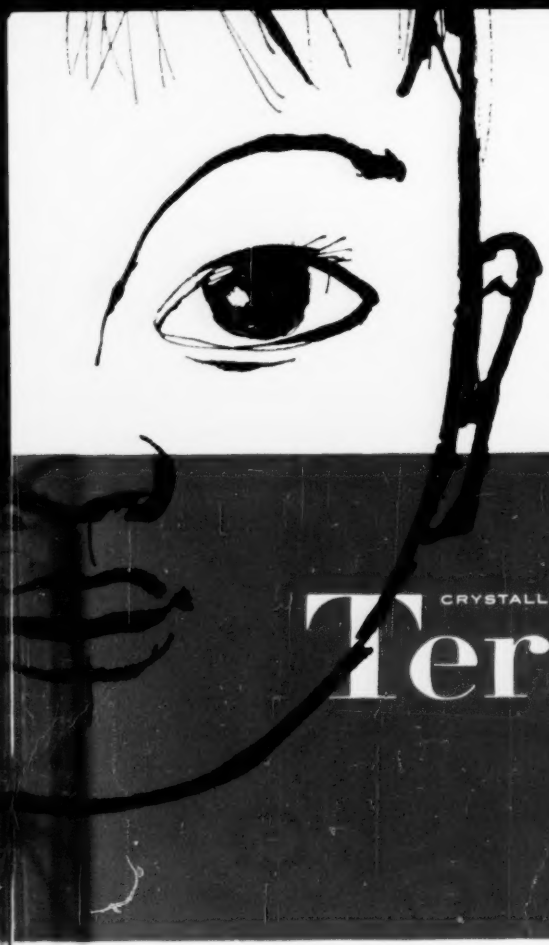

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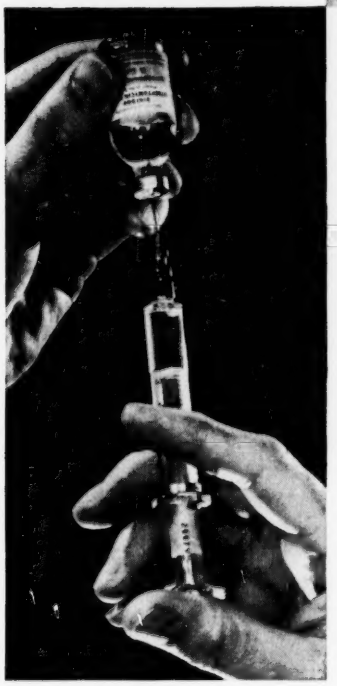

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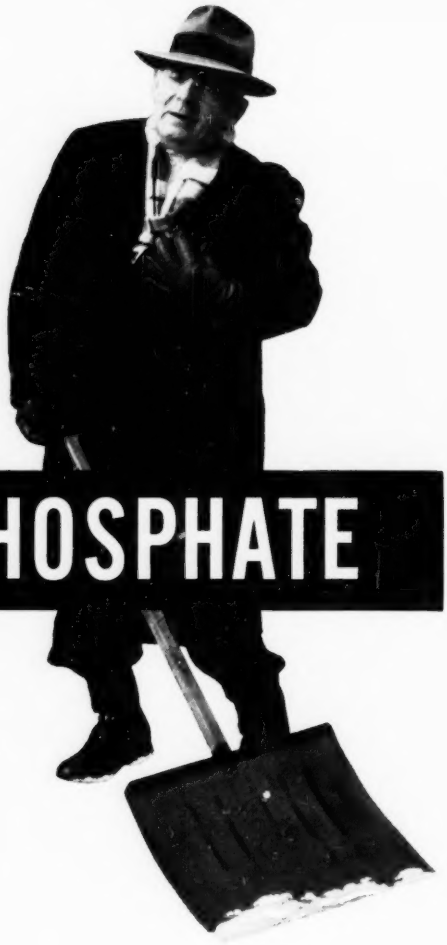

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PHOTOMETRIC MICRODETERMINATION OF HUMAN GAMMA GLOBULIN. I. DEVELOPMENT OF A QUANTITATIVE FLOCCULATION-NINHYDRIN PROCEDURE [1]

By ABRAHAM SAIFER and MICHAEL C. ZYMARIS

(From the Biochemistry Department of the Division of Laboratories, Jewish Sanitarium and Hospital for Chronic Diseases, Brooklyn, N. Y.)

(Submitted for publication April 23, 1951; accepted October 1, 1951)

There are three general categories of procedures employed for the *quantitative* determination of the gamma globulin content of biological fluids. These are (*1*) electrophoretic methods, (*2*) chemical procedures, usually based on salt or organic solvent precipitation, and (*3*) immunochemical methods.

A fourth class of procedures which are *qualitative* or semi-quantitative in nature, are the "protein flocculation reactions." These flocculation procedures are widely used in clinical work to determine changes in protein composition of disease sera, especially in gamma globulin, as compared to normal sera (1).

The application of the electrophoretic technique of Tiselius to biological and medical problems has been fully discussed in recent review articles by Stern and Reiner (2), Luetscher (3) and Gutman (4). From a clinical viewpoint electrophoretic methods have the disadvantages of requiring relatively large amounts of protein material, expensive equipment, and skilled technical help and then permit only a few individual determinations to be performed daily. However, they remain the standard procedure against which other simpler methods are generally compared in the quantitative estimation of a protein fraction.

In recent years three separate salt fractionation procedures have been proposed for the determination of gamma globulin in sera and have been used in clinical investigative work. The methods of Jager and Nickerson (5), Wolfson and his associates (6) and Kibrick and Blonstein (7) have the advantages of requiring relatively small amounts of serum, *i.e.,* 0.5 to 1.0 ml.; of determining the gamma globulin directly on the precipitate obtained by salt fractionation with either ammonium or sodium sulfate; and of then determining its protein content with a colorimetric biuret procedure. The method of Wolfson and his coworkers (6) has been converted into an even simpler turbidimetric method by de la Huerga and Popper (8), and compared by them (9) to the zinc sulfate and the electrophoretic procedures for gamma globulin. However, recovery experiments with added gamma globulin show a range of 73% to 92% with average values of about 85%, which are unsatisfactory results for a quantitative procedure (6, 8).

A number of experimenters have employed alcohol-water mixtures at low temperatures and controlled pH to separate out various protein fractions from human plasma. Pillemer and Hutchinson (10) have employed methanol-water mixtures with acetate buffers to obtain albumin-globulin ratios which conform closely to those obtained with electrophoretic techniques. The most widely used procedures of this kind are the ethanol-buffer methods developed by Edsall (11) and by Cohn and his associates (12–15). Their later procedures are applicable for work with a few milliliters of serum (16, 17) and are especially useful in animal protein fractionation studies, *e.g.,* rat (18), dog (19), and guinea pig (20), where electrophoretic analyses are generally unsatisfactory.

From a clinical viewpoint such procedures are also unsatisfactory in that they require large amounts of blood, *i.e.,* 5 to 20 ml. of plasma, and additional expensive equipment, such as refrigerated centrifuge or water bath; recover less than 90% of the gamma globulin present electrophoretically; and necessitate involved and time-consuming techniques for the isolation and quantitative measurement of any particular component, *e.g.,* gamma globulin.

A number of investigators (21, 22) have proposed the use of immunochemical procedures for

[1] Presented before the Division of Biological Chemistry of The American Chemical Society at the 119th meeting, Boston, Mass., April 15, 1951.

determining gamma globulin in human sera. Such procedures are truly microprocedures in that they can determine 30 to 1,000 μg. of protein accurately and are used to determine quantitatively the gamma globulin content of cerebrospinal fluid as well as sera (22).

The disadvantages of the immunochemical procedure are the involved procedures needed for the preparation and assay of the antisera used, the use of a refrigerated centrifuge and the technical skill and the time required for the involved analyses. In addition Jager and his coworkers (23) have found that the immunological method yields abnormally high values for serum gamma globulin as compared to electrophoretic results.

It is because of the difficulties encountered in the aforementioned procedures for clinical work that Kunkel (24) attempted to use a protein flocculation procedure, *i.e.*, zinc sulfate turbidity, as a *quantitative* measure of serum gamma globulin content. In contrast to the results obtained by Kunkel (24), de la Huerga, Popper, and coworkers (9) report that zinc sulfate turbidity values do not correlate well with either electrophoresis or the protein content of the centrifuged zinc-protein precipitates. They attribute this to the influence of albumin and lipid intake on the zinc sulfate turbidity. These findings were confirmed by Schmid (25) who found that there was no direct relationship between the gamma globulin fraction and the zinc turbidity value when the salt fractionation method of Jager and Nickerson (5) was used to measure the gamma globulin. The lack of correlation encountered in disease cases, where electrophoretic gamma globulin values are known to be high (*e.g.*, multiple myeloma), was even more striking.

Saifer (26) applied the Hanger cephalin-cholesterol flocculation reaction (27) to the semiquantitative determination of the minute amounts of increased gamma globulin present in cerebrospinal fluid in disease cases. In qualitative studies with Cohn's protein fractions, it was shown that flocculation occurs, with the cephalin-cholesterol emulsion in the presence of Hayem's solution (28), with some of the fractions (for instance, fibrinogen or gamma globulin) but not with others (*e.g.*, albumin) under the stated experimental conditions.

The present paper deals with the quantitative aspects of this reaction from the viewpoint of determining the experimental conditions under which gamma globulin could be flocculated out of solution *quantitatively* and the protein content of the complex measured by the ninhydrin reaction of Moore and Stein (29). The effect of the presence of each of the other protein fractions, which together constitute the whole plasma, on the flocculation method for gamma globulin was also investigated quantitatively. This permitted a separation of the fractions into interfering and non-interfering ones and, with the aid of the electrophoretic data, the working out of simple salt fractionation techniques for the elimination of the interfering fractions in applying the method to the microdetermination of serum gamma globulin.

EXPERIMENTAL

A. Reagents and apparatus

1. *Sodium chloride.* 0.85% solution.

2. *Phosphate buffer* (pH 7.00)—0.4 M. Dissolve 39.2 g. anhydrous Na_2HPO_4 and 20.5 g. KH_2PO_4 in a liter of solution. Adjust pH to 7.00 ± 0.02 with a pH meter.

3. *Hayem's solution,* modified (29). Dissolve 10.0 g. NaCl, 11.0 g. Na_2SO_4 (anhydrous) and 2.5 g. $HgCl_2$ in a liter of solution.

4. *Cephalin-cholesterol emulsion.* Prepared from the commercial product supplied by Difco Laboratories, Inc., Detroit, Michigan, according to Hanger (27), as follows: Heat 35 volumes of distilled water in an Erlenmeyer flask to 65° C. Add to the water 1 volume of the ether solution of the cephalin-cholesterol solution, drop by drop with constant agitation, until a homogeneous emulsion is formed. Continue heating the emulsion on a hot plate, at just below the boiling point with occasional shaking, until 30 volumes of the emulsion remain. Cool to room temperature before using. In these experiments a fresh batch of emulsion was prepared for each day's run.

5. *Gamma globulin standard solution.*[2] Weigh out approximately 60 mg. of gamma globulin (Cohn's Fraction II) [3] into a wide-mouth 125 ml. glass bottle. Add 50 ml.

[2] Electrophoretically pure gamma globulin for use as a standard in this procedure may be easily prepared by the ammonium sulfate fractionation method of Kendall as described in the textbook, "Experimental Immunochemistry," by Kabat, E. A., and Mayer, M. M.; Charles C Thomas, Springfield, Ill., 1948, p. 461.

[3] Obtained from the University Laboratory of Physical Chemistry of the Harvard University Medical School through the courtesy of Doctors J. T. Edsall and L. D. Wojick. Fractions Lilly II-1 and Squibb 652 (98–99% gamma globulin) were used in these runs. Fractions used in these experiments were prepared by the older fractionation procedure known as Method 6. Those prepared by the newer Method 10 (15) are apt to contain glycine as a contaminant and must be dialyzed before use.

of 0.85% NaCl *under vacuum conditions,* slowly and with constant agitation until most of the protein goes into solution. Centrifuge off any insoluble material at about 3,000 r.p.m. and transfer the clear supernatant fluid to a glass stoppered bottle. Keep in an icebox at 4° when not in use. The exact protein content of this solution was determined for each day's run by a micro-Kjeldahl digestion and distillation method (30) and diluted with saline to a concentration of about 400 μg. per ml. The optical density values for the standard gamma globulin solution were obtained with 0.00, 0.25, 0.50, and 1.00 ml. aliquots, in quadruplicate, *without* prior flocculation, by the photometric ninhydrin procedure described below, *simultaneously* with each flocculation run so as to establish the level at which the flocculation method gave *quantitative* results.

6. *Ether-reagent grade, anhydrous.*

7. *Sodium hydroxide solution—0.1 N.*

8. *Ninhydrin reagent.* The ninhydrin used in these experiments was obtained from the Dougherty Chemical Company, Jamaica, New York, and twice recrystallized as described by Moore and Stein (29).

a. *Citrate buffer* (pH 5.0)—0.2 M. Dissolve 21.008 g. citric acid, reagent, and 200 ml. of 1 N NaOH in 500 ml. of solution in a volumetric flask. Add a few crystals of thymol as a preservative.

b. *Ethylene glycol monomethyl ether* (*methyl cellosolve*). It was generally found necessary to purify the product obtained from commercial sources by distillation under vacuum to prevent turbid solutions.

c. *Ninhydrin solution.* Dissolve 68 mg. of anhydrous stannous chloride (Flaked Stannochlor,[4] obtained from Metal and Thermit Corporation, New York, N. Y.) in 50 ml. of citrate buffer in a 100 ml. glass stoppered graduated cylinder. Add to this solution 2.0 g. of recrystallized ninhydrin and then 50 ml. of the purified methyl cellosolve. Stopper and dissolve by shaking until all the salt is dissolved. A play of colors occurs during the solution process but the final reagent has a pale yellow color and is water clear. If the solution remains turbid it can usually be clarified by placing the graduate and its contents in warm water. A sufficient amount of this reagent was prepared for each day's run.

9. *Diluent solution.* Mix equal volumes of water and n-propanol, reagent.

10. *Water bath,* constant temperature, 37.5°.

11. *Kahn racks.*

12. *Centrifuge.*

13. *Pan, aluminum,* large 12″ × 18″ × 4″ deep, for boiling water bath.

14. *Spectrophotometer,* Model B Beckman or other similar instrument adaptable for reading small test tubes.

15. *Test tube, cuvettes,* selected—Select 75 × 12 mm. o.d. test tubes, pyrex, without lip, for light absorption characteristics in a manner similar to that described in the paper by Moore and Stein (29).

B. Procedure for the quantitative determination of electrophoretically pure gamma globulin (Cohn's Fraction II)

1. Pipette 0.00 ml., 0.25 ml., 0.50 ml., 0.75 ml. and 1.00 ml. of the standard gamma globulin solution (Fraction II) in quadruplicate, into the selected test tubes, 75 × 12 mm., and then add 0.85% NaCl so that the volume in each tube is 1.00 ml. Add 0.5 ml. of the phosphate buffer (pH 7.00) to each tube, followed by 0.75 ml. of the Hayem's solution and 0.75 ml. of the cephalin-cholesterol emulsion.[5] Mix the contents of each tube by inverting twice, replace in the Kahn racks and place the racks in a water bath at 37.5° for 45 minutes. Centrifuge for 15 minutes at 3,000 r.p.m., using multiple carriers, pour off supernatant fluid, invert tubes in the racks and allow to drain on to cotton gauze placed on the bottom of the racks. Break up the precipitate by tapping the bottom of the tube and add approximately 2 ml. of ether using a 10 or 20 ml. glass syringe. Mix well to wash the precipitate and centrifuge again at 3,000 r.p.m. for five minutes. Aspirate off ether layer carefully without disturbing the protein layer with gentle suction, using a glass pipette drawn out to about a 1/16 inch opening. Repeat washing, centrifugation and aspiration once more. Remove all traces of ether by placing racks with the tubes in the 37.5° water bath for an hour or longer.

2. *Quantitative determination of protein content with photometric ninhydrin procedure.* Add to each tube 1.0 ml. of 0.85% NaCl and 0.2 ml. of 0.1 N NaOH and mix by shaking the rack with the tubes. Add 0.5 ml. of the freshly prepared ninhydrin reagent to each tube, mix the contents well by tapping and place the rack with the tubes in a vigorously boiling water bath for exactly 30 minutes. Mix the contents of each tube by tapping at the end of each 10 minute interval during the heating process. After removal of racks from the water bath allow to cool to room temperature. Add 1.5 ml. of the n-propanol-water diluent to each tube, mix the contents well and centrifuge at 2,000 r.p.m. for five minutes to remove any insoluble material. Clean the outside surface of each tube well and measure the optical density values in a spectrophotometer at 570 mμ, directly in the test tubes, against pure diluent as the blank.

A Beckman Model B spectrophotometer and a Coleman Junior spectrophotometer with test tube adapters were used in these experiments. The results obtained in this run are shown graphically in Figure 1.

[4] When Stannochlor is used in the preparation of the ninhydrin solution in place of the usual reagent $SnCl_2 \cdot 2H_2O$, and the solution prepared *exactly* as described above, it was found that very low and consistent blanks were obtained on day-to-day runs and that no dark colors developed in the solution on prolonged standing even in the presence of air. There is, however, a decrease in color density per unit weight of protein on long standing.

[5] Where large numbers of determinations are to be performed we have found the Cornwall Automatic pipetting units, as recommended in the paper by Dern and Pullman (31), to be both rapid and accurate for the delivery of a constant volume of a solution.

Gamma globulin subfractions II-1, II-2 and II-3 were run in an identical manner and these results are also included in Figure 1.

C. Studies of factors which influence the quantitative flocculation of gamma globulin from solution

1. *Effect of variation of amount of phosphate buffer (0.1 M).* To a series of selected test tubes was added a constant amount of a standardized gamma globulin solution. To each set of four tubes then was added the amount of 0.1 M phosphate buffer (pH 7.00 ± .02) given by each point in Figure 2. The gamma globulin content for the different buffer concentrations was then determined by the quantitative flocculation-ninhydrin procedure exactly as described above.

The results obtained in this run are shown in Figure 2. It is clear that buffer concentration is an important factor in determining whether quantitative flocculation of the gamma globulin takes place. An amount of buffer corresponding to 1.5 ml. of 0.1 M phosphate buffer or greater gave satisfactory quantitative results. In order to reduce the volume of solution used, 0.5 ml. of a 0.4 M phosphate buffer, pH 7.00 ± .02 was employed in all subsequent runs.

2. *Effect of variation of the amount of Hayem's solution.* The variation of this component was studied in a similar manner as was the phosphate buffer described in C 1 above. The results obtained in this run are described graphically in Figure 3.

It should be noted from these data that practically no flocculation occurs in the absence of the Hayem's solution and in the presence of the cephalin-cholesterol emulsion. The method yields quantitative results in the presence of 0.75 ml. or greater of the Hayem's solution.

3. *Effect of the variation of the amount of cephalin-cholesterol emulsion.* The variation of this component was studied in the same manner as were the other two components described above. The results obtained in this run are shown graphically in Figure 4.

A considerable amount of the gamma globulin is flocculated out of solution even in the absence of the emulsion but in the presence of the Hayem's solution. The best quantitative result is obtained at about 0.75 ml. of the emulsion with slightly decreasing gamma globulin values when greater amounts of the emulsion are used.

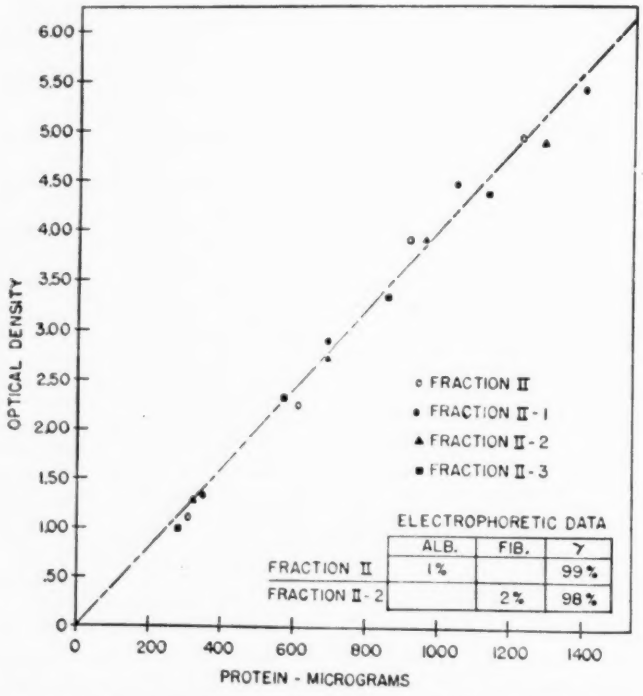

FIG. 1. PHOTOMETRIC DATA FOR GAMMA GLOBULIN (FRACTION II) AND ITS SUBFRACTIONS (FRACTIONS II-1, II-2 AND II-3)

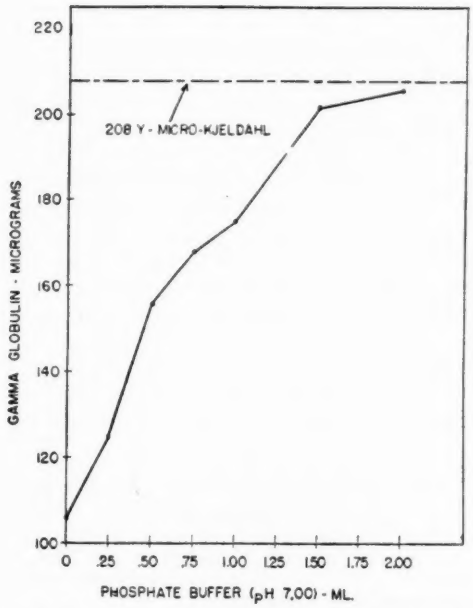

FIG. 2. EFFECT OF VARIATION OF THE AMOUNT OF PHOSPHATE (0.1 M) ON THE QUANTITATIVE FLOCCULATION METHOD FOR GAMMA GLOBULIN

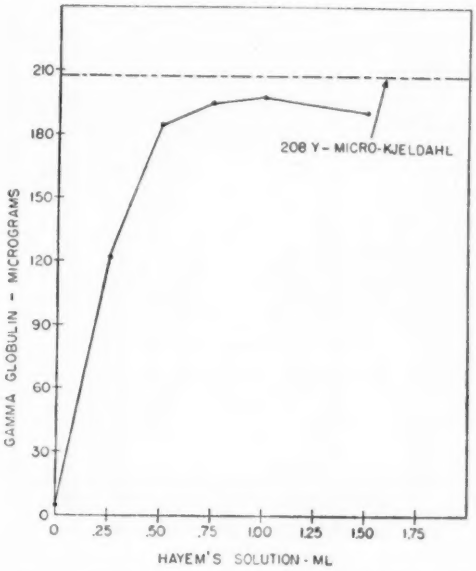

FIG. 3. EFFECT OF VARIATION OF THE AMOUNT OF HAYEM'S SOLUTION ON THE QUANTITATIVE FLOCCULATION METHOD FOR GAMMA GLOBULIN

4. *Effect of variation of the time of standing at 37.5°.* From a large series of preliminary experiments it was found that the temperature at which the flocculation occurs determines to a large extent the rate of flocculation. At room temperature (25°) the rate of flocculation is rather small so that little flocculation occurs within an hour. This rate increases markedly with increasing temperature so that at 37.5°, gamma globulin in the range of 100 to 1000 μg. is completely flocculated after approximately 30 minutes standing or longer. Higher temperatures were not employed to avoid the possibility of denaturation of various protein fractions. The results obtained for the effect of variation of time of standing at 37.5° on quantitative flocculation of the gamma globulin from solution is shown in Figure 5.

5. *Effect of variation of pH of phosphate buffer on the quantitative flocculation of gamma globulin and other protein fractions from solution.* Employing the quantitative flocculation-ninhydrin procedure described above, a large number of experiments were performed with standard gamma globulin solutions to determine the effect of small variations of pH in the vicinity of the reported isoelectric point (15, 23), *i.e.*, pH 7.3. The values given in Table I give the results obtained at ± 0.7 pH units from the isoelectric point. Although the best quantitative results are obtained for pH 7.00, the values obtained for pH 8.00 were sufficiently quantitative to warrant an investigation of the effect of pH variation on the flocculation of the other major protein fractions. As can be observed from the data in Table I, minimum interference from the other fractions also occurs at pH 7.00.

D. Studies of the effect of the presence of other protein fractions on the quantitative determination of gamma globulin by the flocculation-ninhydrin procedure

The fractionation of human plasma by Cohn and his collaborators (12–15) and Edsall (11) has made possible the preparation and characterization of many of the protein components of human plasma. With their original ethanol-water separation technique, human plasma was fractionated into eight major components. The proportion of each component present in the original plasma, and the electrophoretic composition of each fraction, are described in an article by Oncley, Scatchard, and Brown (32). It was these fractions, rather than those prepared with a later fractionation procedure (15), that were investigated for their effect on the quantitative flocculation-ninhydrin method for gamma globulin. In addition a number of subfractions of Fraction II, *i.e.*, II–1, II–2 and II–3, were also determined with this procedure. The electrophoretic data on the bottom of each table are those actually determined on the particular fraction investigated, or those from a similar fraction, as determined at the University Laboratory of Physical Chemistry of the Harvard University Medical School.

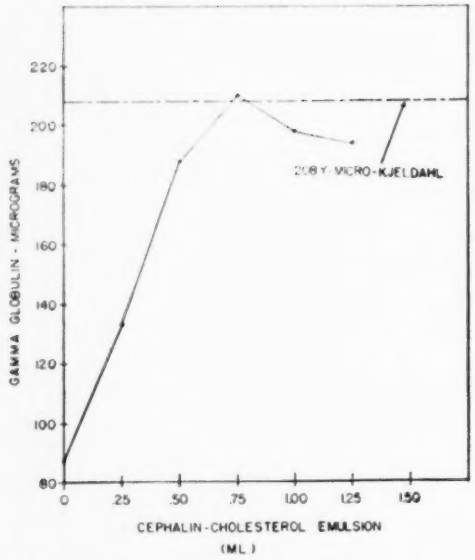

FIG. 4. EFFECT OF VARIATION OF THE AMOUNT OF THE CEPHALIN-CHOLESTEROL EMULSION ON THE QUANTITATIVE FLOCCULATION METHOD FOR GAMMA GLOBULIN

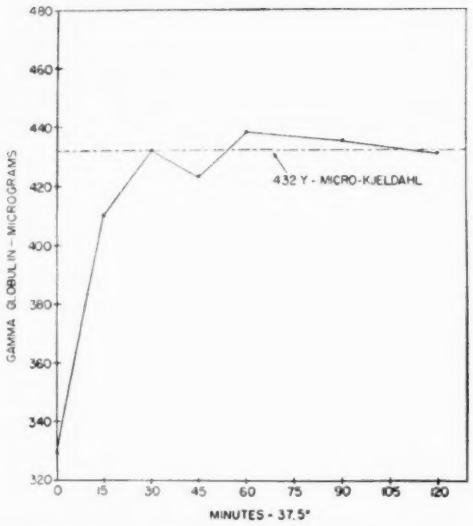

FIG. 5. EFFECT OF THE VARIATION OF THE TIME OF STANDING AT 37.5° ON THE QUANTITATIVE FLOCCULATION METHOD FOR GAMMA GLOBULIN

Employing the quantitative procedure for gamma globulin described above, mixtures of gamma globulin with each of the other protein fractions, *i.e.*, V (albumin), IV-1, IV-4, III-0, III and I (fibrinogen) were determined as if the protein mixtures were pure gamma globulin solutions. The ratio of the particular fraction being tested to the gamma globulin present was varied so as to include the ratio in which these components are present in normal plasma as given in Table VIII. The results obtained in these runs are given in Tables II through VII inclusive. The data on the gamma globulin subfractions are included in Figure 1.

TABLE I

Effect of pH variation on flocculation of Cohn's protein fractions

Protein fraction	1	2	3	4	5	6	7	8	9
	pH 6.60 present	pH 6.60 found	% recovered	pH 7.00 present	pH 7.00 found	% recovered	pH 8.00 present	pH 8.00 found	% recovered
	mg.	mg.		mg.	mg.		mg.	mg.	
Fraction II (Gamma globulin—99%)	224 448	138 290	62 65	219 432	216 432	99 100	219 432	188 406	84 91
Fraction I (Fibrinogen [61%] and antihemophilic globulin)	125	25	20	62.5	6.3	10.1	125	37.5	30
Fraction IV-1 (Alpha$_1$, alpha$_2$, beta$_1$)	189	8	4.2	189	3.7	2.0	189	91	48
Fraction IV-4 (Albumin, alpha$_1$, alpha$_2$, beta$_1$)	405	6	1.5	405	5.1	1.3	405	317	78.2
Fraction V (Albumin—95%)	900	6	0.67	900	20	2.2	900	743	82.5

DISCUSSION OF RESULTS

Study of the various factors which influence the quantitative flocculation-ninhydrin reaction for gamma globulin, as shown in Figures 2 to 5 inclusive, prove that once a critical minimum level of a factor is exceeded, a further increase in the amount of that factor has little influence on the accuracy of the method. Except for the case of the cephalin-cholesterol emulsion, where a definite maximum is indicated, the experimental factor chosen was sufficiently in excess of this critical value to avoid any possibility of error from this source.

Once these critical values were determined the application of the method to the quantitative determination of human gamma globulin (Cohn's Fraction II) was a relatively simple matter. However, quantitative results cannot be obtained unless the precipitated complex is thoroughly washed with anhydrous ether to remove any excess emulsion. A minimum of at least two ether washings was found essential for this purpose.

The data in Figures 3 and 4 indicate that the Hg^{++} ion in Hayem's solution plays a more important role in the flocculation of gamma globulin from solution than does the cephalin-cholesterol emulsion alone. However, *both* are necessary for quantitative results under the given experimental conditions.

Analysis of the complex proves that cholesterol is firmly bound to the protein and is not removed by the ether washings. The exact quantitative composition of this complex is as yet unknown and is being investigated not only for gamma globulin but for the other protein fractions as well. It is sufficient for analytical purposes that the gamma globulin is removed from solution quantitatively and in a form; that is, as part of a complex, which can be easily centrifuged, washed and analyzed all in the original tube. The overall accuracy of such a procedure is ± 5% for 100 to 1,000 μg. of gamma globulin and the optical density values over this range follow the Bouger-Lambert-Beer Law.

The method does not differentiate between gamma globulin (Fraction II) and its subfractions (II–1, II–2 and II–3) and in that respect confirms the findings of Jager and his coworkers (23) and of Kabat and Murray (33) who found these fractions to be immunochemically homogeneous although according to the former investigators these subfractions are inhomogeneous with the ultra-centrifuge and contain particles of different electrophoretic mobility. Cann, Brown, Kirkwood and Hink (34) have separated human gamma globulin into four electrophoretically unique fractions by their electrophoresis-convection technique. In spite of such differences these fractions are all true gamma globulins and any variation in their relative proportions will have no effect on the determination of the total gamma globulin content of a biological fluid by the flocculation-ninhydrin procedure.

TABLE II

Effect of albumin (Fraction V) on quantitative flocculation method for gamma globulin (Fraction II)

Albumin present	Gamma globulin present	Albumin / Gamma glob. present	Estimated gamma globulin recovered	Error	Remarks
μg.	μg.		μg.	%	
	221				Micro-Kjeldahl and Ninhydrin
244	221	1.10	213	−3.6	Ninhydrin
487	221	2.20	216	−2.3	Ninhydrin
732	221	3.30	226	+2.3	Ninhydrin
1,220	221	5.52	236	+6.8	Ninhydrin
1,750	221	7.72	242	+9.5	Ninhydrin

Electrophoretic data—albumin (Fraction V)

Albumin	Alpha1	Alpha2	Beta1
95.5%	2%	1%	0.5%

TABLE III

Effect of Fraction IV-1 on quantitative flocculation method for gamma globulin (Fraction II)

Fraction IV-1 present	Gamma globulin present	Fraction IV-1 / Gamma glob. present	Estimated gamma globulin recovered	Error	Remarks
μg.	μg.		μg.	%	
	199				Micro-Kjeldahl and Ninhydrin
107	199	0.54	185	−7.0	Ninhydrin
214	199	1.08	206	+1.0	Ninhydrin
322	199	1.62	215	+1.1	Ninhydrin
535	199	2.69	188	−5.5	Ninhydrin
749	199	3.76	204	+1.0	Ninhydrin

Electrophoretic data—Fraction IV-1

Alpha1	Alpha2	Beta1
77%	17%	5%

TABLE IV

Effect of Fraction IV-4 on quantitative flocculation method for gamma globulin (Fraction II)

Fraction IV-4 present	Gamma globulin present	Fraction IV-4 / Gamma glob. present	Estimated gamma globulin recovered	Error	Remarks
mg.	mg. 199		mg.	%	Micro-Kjeldahl and Ninhydrin
322	199	1.62	183	−8.0	Ninhydrin
644	199	*3.24*	212	+6.5	Ninhydrin
966	199	4.85	238	+19.5	Ninhydrin
1,610	199	8.08	230	+15.6	Ninhydrin

Electrophoretic data—Fraction IV-4

Albumin	Alpha$_1$	Alpha$_2$	Beta$_1$	Beta$_2$
26%	10%	28%	34%	3%

From the data in Table I it is apparent that accurate control of pH by means of buffers is not only important for quantitative results with gamma globulin itself, but is especially important where gamma globulin is to be determined in the presence of considerable amounts of the other protein fractions, *e.g.*, biological fluids. This is especially apparent in the case of albumin where a change of one pH unit, *i.e.*, from 7.00 to 8.00, causes an increase in the amount of protein flocculated of some 80% as compared to that initially present. Maintenance of the pH below 7.00 gives poorer recoveries of gamma globulin but lessens the interference from albumin. In view of the fact that the albumin fraction inhibits the cephalin-cholesterol flocculation reaction with gamma globulin (1) its elimination or neutralization is a most important consideration.

TABLE V

Effect of Fraction III-0 on quantitative flocculation method for gamma globulin (Fraction II)*

Fraction III-0 present	Gamma globulin present	Fraction III-0 / Gamma glob. present	Estimated gamma globulin recovered	Error	Remarks
mg.	mg. 155		mg.	%	Micro-Kjeldahl and Ninhydrin
53	155	0.34	213	+37.4	Ninhydrin
106	155	0.68	219	+41.3	Ninhydrin
159	155	*1.02*	229	+47.7	Ninhydrin
212	155	1.36	238	+53.5	Ninhydrin

Electrophoretic data—Fraction III-0†

Alpha	Beta	Gamma	Fibrin.
8.3%	78.5%	5.0%	8.3%

* According to personal communication from Dr. L. D. Wojcik of the Harvard University Laboratory of Physical Chemistry, the Fraction III-0 used in these experiments is composed chiefly of beta-lipoprotein with a small amount of beta globulin.

† From data by Oncley, Scatchard, and Brown (32).

TABLE VI

Effect of Fraction III on quantitative flocculation method for gamma globulin (Fraction II)

Fraction III present	Gamma globulin present	Fraction III / Gamma glob. present	Estimated gamma globulin recovered	Error	Remarks
mg.	mg. 155		mg.	%	Micro-Kjeldal and Ninhydrin
106	155	*0.69*	240	+54.8	Ninhydrin
212	155	1.38	298	+92.5	Ninhydrin
318	155	2.07	346	+123	Ninhydrin
424	155	2.76	396	+155	Ninhydrin

*Electrophoretic data—Fraction III**

Alpha	Beta	Fibrin.	Gamma
7.0%	55.6%	26.2%	11.2%

* From data by Oncley, Scatchard, and Brown (32).

The data in Tables II to VII deal with the effect of all the other fractions, as separated by Cohn's Method 6, which constitute the whole plasma. The non-interfering fractions are Fractions V, IV–1, IV–4 and VI. The latter fraction was not tested and is listed as a non-interfering fraction because it contains only a small amount of protein

TABLE VII

Effect of Fraction I (fibrinogen and antihemophilic globulin) on quantitative flocculation method for gamma globulin (Fraction II)

Fraction I present	Gamma globulin present	Fraction I / Gamma glob. present	Estimated gamma globulin recovered	Error	Remarks
mg.	mg. 240		mg.	%	Micro-Kjeldahl and Ninhydrin
176.5	240	0.32	266	+10.8	Ninhydrin
253	240	*0.64*	288	+20.0	Ninhydrin
330	240	0.96	320	+33.3	Ninhydrin
06	240	1.27	350	+46.0	Ninhydrin

Electrophoretic data—Fraction I

Albumin	Alpha$_1$	Beta$_1$	Fibrin.	Gamma
7%	8%	15%	61%	9%

which is mostly albumin. From the electrophoretic viewpoint the non-interfering fractions are either rich in albumin or alpha protein and contain less than 37% of beta globulin. At the ratio of the particular protein fraction to gamma globulin found in normal plasma (see Table VIII), these fractions when run together with gamma globulin, do not give values more than 5% greater than that due to gamma globulin alone.

The interfering fractions are I, III and III–0 and the amounts of each present in normal plasma and their ratio to gamma globulin are also given in Table VIII. These fractions are rich in fibrinogen or beta globulin (or beta lipoprotein) and introduce considerably positive errors when run together with gamma globulin. As shown in Tables V–VII these errors decrease rapidly with decreasing protein fraction/gamma globulin ratio, and indicate the possibility of eliminating such errors by reducing the concentrations of these components by salt fractionation prior to the flocculation procedure.

Table VIII contains data for the amount of each protein fraction present in normal plasma, the ratio of each fraction to the gamma globulin present in plasma and a summary of the predicted errors for plasma and sera as calculated from the preceding tables, Tables II to VII inclusive. In making these calculations it was assumed that any gamma globulin shown to be present in the electrophoretic data, given at the bottom of each table, would be included in the analysis of sera or plasma for gamma globulin with the flocculation-ninhydrin method. In addition it was assumed that any fibrinogen present in plasma would be removed as an interfering factor during the blood-clotting process and would not be present in sera. On the basis of these assumptions it was calculated that an error of about + 82% would occur if plasma were analyzed by the flocculation-ninhydrin method for gamma globulin. The error for sera was estimated to be about + 48%.

The elimination of these large positive errors for serum gamma globulin determinations, the comparison of the quantitative flocculation-ninhydrin method with electrophoretic data on the same sera, and the evaluation of a *new clinical entity*, the $\frac{\text{(total serum flocculation-ninhydrin value)}}{\text{(globulin clot flocculation-ninhydrin value)}}$ for both normal and disease cases is discussed in the

TABLE VIII

Summation of data on the effect of Cohn's protein fractions on the quantitative flocculation method for gamma globulin (Fraction II)

Plasma protein fraction	Estimated amount of protein fraction in 1 ml. of a 1:50 diltn. of normal plasma*	Prot. fraction / Gamma glob.	Predicted error† from previous data—plasma	Remarks
	μg.		μg.	
Fraction II (Gamma globulin)	120		±6	With colorimetric ninhydrin method.
Fraction I (Fibrinogen, etc.)	68	0.57	+(5–17) Sera = 0	Contains 9% gamma glob. fibrinogen removed during blood clotting.
Fraction III (Fibrinogen, beta globulin, etc.)	80	0.67	+(34–43) Sera = +(17–25)	Contains 11.2% gamma and 26.2% fibrinogen.
Fraction III-0 (Beta lipoprotein, beta globulin)	108	0.98	+(42–54) Sera = +(31–43)	Contains 5% gamma and 8.3% fibrinogen.
Fraction IV-1 (Alpha$_1$ and alpha$_2$ globulins)	104	0.87	0	Presence of these fractions, at indicated ratio, gives gamma globulin values within experimental limits of error of method.
Fraction IV-4 (Albumin, alpha$_2$ and beta$_1$ globulins)	116	0.97	0	
Fraction V (Albumin)	632	5.3	0	
Fraction VI (Albumin, etc.)	18	0.15	0	Not tested. The only protein present in significant amounts is albumin.

* Calculated from data by Oncley, Scatchard, and Brown(32).
† Predicted error: for Plasma = + 87 μg. (aver.) or +82.5%;
for Sera = 58 μg. (aver.) or +48.3%.

subsequent paper. The application of the general principles of quantitative protein flocculation for the microdetermination of other electrophoretic components besides gamma globulin, is presently being investigated in this laboratory, *e.g.*, albumin and fibrinogen.

SUMMARY

1. A simple accurate photometric microprocedure for the quantitative determination of electrophoretically pure gamma globulin (Cohn's Fraction II) in the range of 100 to 1,000 μg. of protein, has been described.

2. The principle of the method involves the quantitative flocculation from solution of the gamma globulin with a cephalin-cholesterol emulsion in the presence of Hayem's solution and determination of the protein content of the ether washed precipitate photometrically with the ninhydrin reaction, all in the same tube.

3. The various factors which influence the quantitative flocculation-ninhydrin reaction, *e.g.*, pH, temperature, time, concentration of the reagents, etc., have been carefully studied and controlled.

4. Subfractions of gamma globulin gave results which were identical with those obtained with the original whole Fraction II.

5. The effect of the presence of each of Cohn's other protein fractions (Method 6) which constitute whole plasma, have been investigated for their effect on the quantitative determination of gamma globulin. It was found experimentally that only those fractions which are rich in fibrinogen, beta globulin or beta lipoprotein interfere.

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REFERENCES

1. Maclagan, N. F., Flocculation tests; chemical and clinical significance. Brit. Med. J., 1948, **2**, 892.
2. Stern, K. G., and Reiner, M., Electrophoresis in medicine. Yale J. Biol. & Med., 1946, **19**, 67.
3. Luetscher, J. A., Jr., Biological and medical applications of electrophoresis. Physiol. Rev., 1947, **27**, 621.
4. Gutman, A. B., Plasma proteins in disease, *in* Advances in Protein Chemistry, edited by Anson, M. L., and Edsall, J. T. Academic Press, Inc., New York, 1948, Vol. IV, p. 155.
5. Jager, B. V., and Nickerson, M., Simple quantitative chemical method for estimating gamma globulin in human serum. J. Biol. Chem., 1948, **173**, 683.
6. Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F., Studies in serum proteins; rapid procedure for estimation of total protein, true albumin, total globulin, alpha globulin, beta globulin and gamma globulin, in 1.0 ml. of serum. Am. J. Clin. Path., 1948, **18**, 723.
7. Kibrick, A. C., and Blonstein, M., Fractionation of serum into albumin and alpha, beta and gamma globulin by sodium sulphate. J. Biol. Chem., 1948, **176**, 983.
8. de la Huerga, J., and Popper, H., Estimation of serum gamma globulin concentration by turbidimetry. J. Lab. & Clin. Med., 1950, **35**, 459.
9. de la Huerga, J., Popper, H., Franklin, M., and Routh, J. I., Comparison of the results of gamma globulin and zine sulphate turbidity test with electrophoretic determination of the gamma globulins. J. Lab. & Clin. Med., 1950, **35**, 466.
10. Pillemer, L., and Hutchinson, M. C., The determination of the albumin and globulin contents of human serum by methanol precipitation. J. Biol. Chem., 1945, **158**, 299.
11. Edsall, J. T., The plasma proteins and their fractionation, *in* Advances in Protein Chemistry, edited by Anson, M. L., and Edsall, J. T. Academic Press, Inc., New York, 1947, Vol. III, p. 383.
12. Cohn, E. J., History of plasma fractionation *in* Advances in Military Medicine. Little, Brown & Co., Boston, 1947, Vol. I, Chap. 28, p. 364.
13. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J. Am. Chem. Soc., 1946, **68**, 459.
14. Cohn, E. J., Directions for the Preparation of Prothrombin, Isoagglutinins and Serum Gamma Globulin (Human), Method 9. Mimeographed pamphlet, Lab. Phys. Chem., Harvard U. Medical School, Boston, Revision of July 27, 1945.
15. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E., A system for the separation of the components of human blood: quantitative procedure for the separation of the protein components of human plasma. J. Am. Chem. Soc., 1950, **72**, 465.

16*a*. Pearsall, H. R., and Chanutin, A., Electrophoretic, nitrogen and lipide analyses of plasma and plasma fractions of healthy young men. Am. J. Med., 1949, **7**, 297.

b. Pearsall, H. R., and Chanutin, A., Electrophoretic, nitrogen and lipide analyses of plasma and plasma fractions in disease. *Ibid.*, 301.

17. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schultz, E. L., Chemical, clinical and immunological studies on the products of human plasma fractionation. XL. Quantitative separation and determination of the protein components in small amounts of normal human plasma. J. Clin. Invest., 1951, **30**, 99.

18. Gjessing, E. C., and Chanutin, A., Electrophoretic study of plasma and plasma fractions of normal and injured rats. J. Biol. Chem., 1947, **169**, 657.

19. Gjessing, E. C., Ludewig, S., and Chanutin, A., Fractionation, electrophoresis and chemical studies of proteins in sera of control and injured dogs. J. Biol. Chem., 1947, **170**, 551.

20. Ecker, E. E., and Likover, B., A micromethod for the determination of the albumin-globulin ratio in guinea pig serum. J. Lab. & Clin. Med., 1947, **32**, 1500.

21. Kendall, F. E., Studies on serum proteins. I. Identification of a single serum globulin by immunological means; its distribution in sera of normal individuals and of patients with cirrhosis of the liver and with chronic glomerulonephritis. J. Clin. Invest., 1937, **16**, 921.

22*a*. Kabat, E. A., Glusman, M., and Knaub, V., Quantitative estimation of albumin and gamma globulin in normal and pathologic cerebrospinal fluid by immunochemical methods. Am. J. Med., 1948, **4**, 653.

b. Kabat, E. A., Glusman, M., and Knaub, V., Immunochemical estimation of albumin and gamma globulin in normal and pathologic cerebrospinal fluid. Federation Proc., 1948, **7**, 306.

23. Jager, B. V., Smith, E. L., Nickerson, M., and Brown, D. M., Immunological and electrophoretic studies on human gamma globulins. J. Biol. Chem., 1948, **176**, 1177.

24. Kunkel, H. G., Estimation of alterations of serum gamma globulin by turbidimetric technique. Proc. Soc. Exper. Biol. & Med., 1947, **66**, 217.

25. Schmid, R., The zinc turbidity test and its clinical application. J. Lab. & Clin. Med., 1950, **36**, 52.

26. Saifer, A., Estimation of increased gamma globulin and fibrinogen in cerebrospinal fluid; a serial dilution-flocculation method. J. Lab. & Clin. Med., 1950, **36**, 130.

27. Hanger, F. M., Flocculation of cephalin-cholesterol emulsions by pathological sera. Tr. A. Am. Physicians, 1938, **53**, 148.

28. Mandel, E. E., and Paris, D. A., Evaluation of the flocculation test with Hayem's solution. J. Lab. & Clin. Med., 1949, **34**, 653.

29. Moore, S., and Stein, W. H., Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem., 1948, **176**, 367.

30. Markham, R., Steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J., 1942, **36**, 790.

31. Dern, R. J., and Pullman, T. N., The accuracy, precision and utility of the syringe as a pipetting device. J. Lab. & Clin. Med., 1950, **36**, 494.

32. Oncley, J. L., Scatchard, G., and Brown, A., Physicochemical characteristics of certain of the proteins of normal human plasma. J. Phys. & Coll. Chem., 1947, **51**, 184.

33. Kabat, E. A., and Murray, J. P., A comparison of human gamma globulins in their reactivity with rabbit anti-gamma globulin by the quantitative precipitin method. J. Biol. Chem., 1950, **182**, 251.

34. Cann, J. R., Brown, R. A., Kirkwood, J. G., and Hink, J. H., Jr., Fractionation of human immune gamma globulin. J. Biol. Chem., 1950, **185**, 663.

PHOTOMETRIC MICRODETERMINATION OF HUMAN GAMMA GLOBULIN. II. COMPARISON OF QUANTITATIVE FLOCCULATION-NINHYDRIN METHOD WITH ELECTROPHORETIC METHOD[1]

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The main purpose in performing the detailed experimental studies in Part I is the application of the photometric method to the determination of the gamma globulin content of human sera. These experimental studies show that protein fractions such as albumin or alpha globulins, whose electrophoretic mobilities are far removed from gamma globulin, cause little interference with its determination. Whereas those fractions, such as beta globulins or fibrinogen, whose electrophoretic mobilities are close to that of gamma globulin, are apt to cause positive errors, unless their concentrations are reduced below a certain *critical ratio*, as compared to gamma globulin. This furnishes experimental evidence that there is a relationship between mobility and flocculation of protein fractions which is to be expected from theoretical considerations (1).

A similar close parallelism between mobility and solubility of proteins in concentrated salt solution, *e.g.*, ammonium sulfate, has been pointed out by Svensson (2) and Pedersen (3). Svensson concludes that it is not a simple matter to isolate pure electrophoretic components by salt fractionation. His results were confirmed by the careful experimental studies of Majoor (4) on the solubility of protein fractions in various concentrations of sodium sulfate in comparison with results of electrophoresis experiments. His results show that particularly in the case of disease sera, a wide range of concentrations of sodium sulfate is required for complete precipitation of gamma globulin. He states that at any single salt concentration there is undeniable overlapping of protein fractions and no absolute separation takes place. The experimental technique in this paper conforms closely to that of Majoor in that a salt fractionation procedure is used which will precipitate quantitatively gamma globulin under *all* conditions with minimum contamination from the other protein fractions. Since the salt fractionation procedure is performed prior to the flocculation method, it results in a sufficient removal of the interfering fractions so as to make the method quantitative solely for gamma globulin.

In spite of certain difficulties with electrophoretic separation of protein fractions, particularly in disease sera, *e.g.*, nephrosis, in which lipids are bound to the globulin fractions (5), it still remains the standard procedure against which other quantitative methods are compared. A large series of normal and disease sera were run comparatively with both the photometric flocculation-ninhydrin procedure and the electrophoretic method. The results obtained prove conclusively that the photometric flocculation-ninhydrin method provides a simple, accurate micro-procedure for the quantitative estimation of the gamma globulin content of human sera in both health and disease.

In addition, the flocculation-ninhydrin method furnishes a simple technique for the quantitative estimation of another protein fraction present in the whole sera which is probably a slower migrating beta globulin or beta lipoprotein. The ratio of this component to the gamma globulin, as estimated with the flocculation-ninhydrin method, furnishes a *new index* of *disease* which remains relatively constant for normal sera but which rises in cases of nephrosis and falls in cases of hepatitis or multiple myeloma. Unlike more complicated methods, the quantitative flocculation-ninhydrin procedures described in this paper can be used for large scale clinical evaluation of gamma globulin changes in disease, *e.g.*, tuberculosis sera. It should also prove useful in supplementing the elec-

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trophoretic procedure in obtaining quantitative results where electrophoretic patterns are difficult to interpret as in cases of nephrosis, and multiple myeloma, and with certain animal sera. The described procedures should prove especially applicable to clinical studies in laboratories where electrophoretic or immunochemical methods are presently unavailable.

EXPERIMENTAL

A. Reagents and apparatus

In addition to those described in the previous paper, the following are required:

1. *Sodium sulfite*—20% solution. Dissolve 200 g. of anhydrous sodium sulfite, reagent grade, in distilled water by vigorous shaking at room temperature and dilute to a liter in a graduated cylinder.

2. *Sodium chloride solution*—11% solution.

3. *Cellophane tubing*, 1 inch wide, obtained from Visking Corporation, Chicago, Illinois.

B. Method for determination of gamma globulin content of human serum

1. *Photometric flocculation-ninhydrin procedure for dialyzed globulin clot as gamma globulin.* To 7.5 ml. of 20% sodium sulfite in a 15 ml. graduated centrifuge tube, add 0.5 ml. of clear serum. Mix contents well and let stand 20 minutes or longer at room temperature. Add approximately 5 ml. of anhydrous ether, stopper with a clean rubber stopper and shake vigorously for 30 seconds. Centrifuge at about 2,500 r.p.m. for about 10 minutes. Remove tube from centrifuge and tilt gently to an almost horizontal position so that the globulin button at

TABLE I

Normal sera: Comparison of electrophoretic data with the flocculation-ninhydrin method

Case No.	Electrophoretic data							Photometric data*			Electrophoretic
	T. P. g. %	Alb. g. %	Glob. g. %	Alpha$_1$ g. %	Alpha$_2$ g. %	Beta g. %	Gamma g. %	Gamma glob. clot g. %	Gamma glob. tot. serum g. %	Total serum / Glob. clot	Beta +gamma / Gamma
1	7.6	4.6	3.0	0.4	0.7	1.0	0.9	0.96	—	—	2.1
2	7.7	4.7	3.0	0.3	0.6	1.0	1.1	1.25	—	—	2.0
3	6.7	3.6	3.1	0.4	0.8	0.8	1.0	0.94	—	—	1.8
4	6.6	3.5	3.1	0.4	0.7	0.7	1.2	0.96	1.43	1.49	1.6
5	7.5	4.0	3.5	0.4	0.7	1.1	1.2	1.25	1.82	1.46	1.9
6	7.0	4.6	2.4	0.2	0.6	0.8	0.9	1.05	1.59	1.51	1.9
7	6.8	3.6	3.2	0.4	0.7	1.2	0.9	1.09	1.55	1.42	2.3
8	7.9	4.3	3.6	0.5	0.7	1.2	1.2	1.14	1.64	1.42	2.0
Mean	7.2	4.1	3.1	0.4	0.7	1.0	1.1	1.08	1.61	1.46	2.0

Summary of data for normal sera run with the flocculation-ninhydrin method

No. of sera	Gamma glob. clot g. %		Gamma glob. tot. serum g. %		Gamma glob. (tot. serum) / Gamma glob. (glob. clot)	
	Range	Mean	Range	Mean	Range	Mean
32	0.72–1.25	1.00	1.09–2.00	1.54	1.40–1.83	1.55

Typical results for the flocculation-ninhydrin method

Case no.	Glob. clot (1:50 diltn.) ml.	Gamma glob. clot g. %	Standard deviation from mean
100	0.50	1.34; 1.34; 1.27.	±.040
	1.00	1.28; 1.34; 1.27.	
		Aver.: 1.30	
101	0.50	0.78; 0.85; 0.79.	±.031
	1.00	0.86; 0.84; 0.85.	
		Aver. = 0.83	

Recovery of added gamma globulin (Fraction II)

No. of sera	Gamma glob. added μg.	Average gamma glob. recovered* μg.	Average error %
6	173	188	+8.7
6	288	299	+3.5
8	310	312	+0.6

* Each value given for the flocculation-ninhydrin method is the average of six determinations at two concentrations.

the bottom of the ether layer floats away from the side of the tube and permits access to a capillary-tipped pipette to the bottom of the tube. Remove all the liquid with gentle suction taking care not to disrupt the globulin clot. Add about 3 ml. of distilled water and suspend the protein by vigorous shaking of the tube.

Prepare cellophane bags by cutting 6 inch strips of cellophane tubing and soaking in water for about five minutes. Open one end of the tubing, then twist this end several times and tie off this end with a double knot. Separate the other end of the tubing and open wide by blowing into it. Transfer the 3 ml. of the protein suspension to the cellophane bag and rinse out centrifuge tube with three 2 ml. portions of distilled water, and transfer the washings quantitatively to the cellophane bag. Wash down inside of cellophane bag with another 2 ml. portion of distilled water and tie off the open end with a double knot leaving only a small amount of air space. Label bags by stapling on tag and dialyze out all soluble material from the protein solution by placing bag in a container and running cold tap water over it for 12 hours or longer. Remove bag from water bath, cut open one end of the bag and transfer contents quantitatively with distilled water to 25 ml. volumetric flasks keeping total volume of solution and washings below 20 ml. Add 2 ml. of 11% sodium chloride solution, dilute to 25 ml. mark with distilled water and mix.

Pipette three 0.50 ml. aliquots and three 1.00 ml. aliquots into selected test tubes (75 × 12 mm.) and then add 0.85% NaCl where necessary, so that the volume in each tube is exactly 1.00 ml.

2. Photometric flocculation-ninhydrin procedure for total serum as gamma globulin. Prepare a 1:50 dilution of same serum with 0.85% NaCl in a volumetric flask. Pipette three 0.5 ml. aliquots and three 1.00 ml. aliquots of the diluted serum into selected test tubes (75 × 12 mm.) and then add 0.85% NaCl, where necessary, so that the volume in each tube is exactly 1.00 ml.

3. Standards and blanks. Pipette in triplicate 0.5 ml. and 1.00 ml. aliquots of pure gamma globulin standard solution, prepared as described in Section A-5 of the preceding paper, into selected test tubes and then add 0.85%

TABLE II

Disease sera: Effect of increased alpha globulins (electrophoretic) on the flocculation-ninhydrin method

Electrophoretic data								Photometric data			Electrophoretic	Diagnosis
Case no.	T. P. g. %	Alb. g. %	Glob. g. %	Alpha$_1$ g. %	Alpha$_2$ g. %	Beta g. %	Gamma g. %	Gamma glob. clot g. %	Gamma glob. tot. ser. g. %	Tot. ser. / Glob. clot	(Beta + gamma) / Gamma	
34	7.3	2.1	5.2	1.3	1.7	1.1	1.0	1.13	—	—	2.1	Reticulocell sarcoma (4/12)
35	7.9	3.3	4.6	1.0	1.7	0.9	1.0	1.14	—	—	1.9	Same case (4/25)
36	7.2	2.5	4.7	1.0	1.7	1.1	1.0	1.13	1.78	1.48	2.1	Same case (4/29)
37	6.8	3.4	3.4	0.4	1.0	0.9	1.0	1.10	1.59	1.45	1.9	Possible T.B.
38	6.8	3.0	3.8	0.6	1.2	1.2	0.9	1.11	1.69	1.52	2.3	Collagen disease
39	7.5	3.6	3.9	0.6	1.1	1.1	1.1	1.27	1.87	1.47	2.0	Rheumatic fever, acute
40	7.8	4.0	3.8	0.6	0.9	1.2	1.1	0.98	1.75	1.79	2.1	Rheumatoid arthritis
41	7.2	3.3	3.9	0.6	1.1	1.2	1.0	1.02	1.62	1.58	2.2	Unknown
Mean	7.3	3.2	4.2	0.86	1.3	1.1	1.0	1.11	1.70	1.55	2.1	
Increased alpha + gamma globulins												
42	7.4	2.5	4.9	0.9	1.6	1.2	1.3	1.36	1.84	1.35	1.9	Lupus erythematosus, pneumon.
43	7.5	3.0	4.5	0.5	1.2	1.2	1.5	1.51	1.78	1.18	1.8	Lupus erythematosus
44	7.9	3.0	4.9	0.8	1.5	1.1	1.4	1.50	2.04	1.36	1.8	Unknown
Increased alpha and decreased gamma globulins												
45	6.8	4.1	2.7	0.3	0.9	0.8	0.7	0.79	1.69	2.14	2.2	Baby—21 days old
Increased alpha and decreased beta + gamma globulins												
46	4.8	1.3	3.5	0.4	2.0	0.6	0.5	0.65	1.25	1.92	2.2	Nephrotic syndrome
Increased alpha + beta + gamma globulins												
47	7.9	3.5	4.4	0.4	1.1	1.4	1.5	1.45	2.17	1.49	1.9	Lupus erythematosus
48	7.5	2.6	4.9	0.7	1.1	1.2	2.0	2.06	2.52	1.22	1.1	Unknown

TABLE III

Disease sera: Effect of increased beta globulins (electrophoretic) on the flocculation-ninhydrin method

Electrophoretic data								Photometric data			Electrophoretic	Diagnosis
Case no.	T. P. g. %	Alb. g. %	Glob. g. %	Alpha$_1$ g. %	Alpha$_2$ g. %	Beta g. %	Gamma g. %	Gamma glob. clot g. %	Gamma glob. tot. ser. g. %	Tot. ser. / Glob. clot	(Beta+gamma) / Gamma	
49	7.3	3.6	3.7	0.5	0.8	1.3	1.1	0.93	1.61	1.73	2.2	Unknown
50	7.2	3.9	3.3	0.3	0.6	1.4	1.1	1.26	1.85	1.47	2.3	Lupus erythematosus
51	7.8	4.5	3.3	0.2	0.8	1.5	0.9	0.98	1.44	1.47	2.6	Unknown
52	7.5	3.4	4.1	0.4	0.8	2.0	0.9	0.92	1.46	1.59	3.2	Multiple myeloma?
Mean	7.5	3.9	3.6	0.4	0.8	1.6	1.0	1.02	1.59	1.57	2.6	
Increased alpha + beta globulins												
53	7.2	3.2	4.0	0.4	0.8	1.4	1.4	1.30	1.98	1.52	2.0	Pregnancy 9 months
54	7.0	3.0	4.0	0.4	0.9	1.3	1.4	1.34	1.79	1.34	1.9	Diabetes
55	7.0	2.9	4.1	0.5	0.9	1.3	1.4	1.33	1.75	1.32	1.9	Same case—serum drawn 1 hour later
Increased gamma + beta globulins												
56	7.6	3.7	4.1	0.4	0.7	1.3	1.6	1.58	2.20	1.40	1.8	Sarcoidosis
57	8.2	1.9	6.3	0.6	0.5	2.1	3.1	2.96	3.33	1.13	1.7	Hodgkin's disease
Increased beta and decreased gamma globulins												
58	7.0	4.3	2.7	0.2	0.7	1.2	0.6	0.72	1.45	2.02	3.0	Slight polyerythemia
59	No results obtainable from electrophoretic pattern							0.52	1.99	3.82	—	Lipoid nephrosis Total cholesterol = 772 mg.%
60	4.2	0.5	3.7	3.2			0.5?	0.18	1.10	6.12	—	Lipoid nephrosis Total cholesterol = 499 mg. %

NaCl where necessary so that the volume in each tube is exactly 1.00 ml. For blank tubes, pipette in triplicate 1.00 ml. aliquots of 0.85% NaCl into the selected test tubes. The remainder of the procedure, including the flocculation and washing of the protein precipitate and the development of the color with the ninhydrin reaction, is exactly as described under Section B of the preceding paper. Blanks, standard and unknowns, are run simultaneously under identical conditions and the spectrophotometric readings and calculations performed in the usual manner according to the Bouger-Lambert-Beer Law.

C. Comparison of the flocculation-ninhydrin procedure with the electrophoretic method for gamma globulin

Fifty sera, from both normal and disease cases were analyzed with the photometric flocculation-ninhydrin method and the electrophoretic method independently in two different laboratories.[2] The instrument used for the electrophoretic determinations is the Tiselius electrophoresis apparatus. Patterns were obtained by a modification of the scanning method of Longsworth (6). Each result is the mean of two measurements. Each pattern was enlarged to three times its actual size, was traced and the areas measured with a planimeter. Areas were defined by the method of Longsworth (7). In all cases the data were computed from the ascending pattern as it gives better resolution and has no beta anomaly. Barbiturate buffer at pH 8.6 and 0.1 ionic strength was used for these runs and the power was adjusted so that it was always equal to 2 watts.

To prepare serum for electrophoretic analysis, 1.0 ml. of serum was added to 3.0 ml. of buffer and dialyzed against 300 ml. of buffer for 17 hours at 4° C. All analyses were made in the Tiselius microcell of 2 ml. capacity. The various protein components expressed as percentages are converted into absolute values, *i.e.*, grams %, from the total protein value as determined by a micro-Kjeldahl digestion-steam distillation procedure.

The results obtained in the comparative study with the two methods are given in Tables I through IV. The flocculation-ninhydrin values given for both the dialyzed globulin clot and diluted whole sera represent triplicate determinations at two different concentrations, *i.e.*, 0.50 and 1.00 ml. aliquots of a 1 : 50 dilution of the original se-

[2] The electrophoretic determinations for this paper were performed by Mr. Henry Berger of the Mattie Billings Lowe Laboratory of the Jewish Hospital of Brooklyn.

rum. These values were obtained without prior knowledge of the electrophoretic values for gamma globulin. Some typical results as obtained for two sera, one low and the other high in gamma globulin, are included in Table I so as to indicate the reproducibility of the procedure. Some data on the recovery of pure gamma globulin added to sera are also included in Table I to show the accuracy of the method.

Thirty-two normal sera obtained from the hospital staff were run with the flocculation-ninhydrin method alone. The subjects used were adults in good health, from 20 to 50 years of age and about equally distributed as to sex. The summary of the results obtained for these normal sera is given in Table I. These results are compared with those obtained electrophoretically by Reiner, Fenichel, and Stern (8) in Table V for a similar series.

Twenty-five sera from hospitalized tuberculosis patients were run by the flocculation-ninhydrin method. These results were compared with those obtained electrophoretically by Seibert, Pfaff, and Seibert (9) for a similar group of tuberculosis patients.

The rest of the comparison experiments between the photometric flocculation-ninhydrin procedure and the electrophoretic method are divided into tables based upon the increase in a particular electrophoretic globulin component, *e.g.*, Table II (increased alpha globulins), Table III (increased beta globulins) and Table IV (increased gamma globulins). Table V summarizes all the preceding data and gives a statistical analysis of the results obtained with the electrophoretic and flocculation-ninhydrin methods for both normal and disease cases.

DISCUSSION

The comparative data between the electrophoretic method and the photometric flocculation-ninhydrin methods are shown in Tables I through V inclusive. These data prove conclusively that the globulin clot-gamma globulin values are equivalent to electrophoretic gamma globulin values over a wide range of values for both normal and

TABLE IV

Disease sera: Effect of increased gamma globulins (electrophoretic) on the flocculation-ninhydrin method

Electrophoretic data								Photometric data			Electrophoretic	Diagnosis
Case no.	T. P. g. %	Alb. g. %	Glob. g. %	Alpha$_1$ g. %	Alpha$_2$ g. %	Beta g. %	Gamma g. %	Gamma glob. clot g. %	Gamma glob. tot. ser. g. %	Tot. ser. / Glob. clot	(Beta + gamma) / Gamma	
61	7.3	4.0	3.3	0.3	0.7	1.1	1.3	1.32	1.93	1.46	1.8	Rheumatoid arthritis
62	7.0	3.7	3.3	0.3	0.5	1.1	1.3	1.47	2.06	1.40	1.9	Idiopathic uveitis
63	6.8	3.2	3.6	0.4	0.6	1.1	1.5	1.72	2.38	1.38	1.7	Psoriasis; arthritis of cervical spine
64	7.5	4.2	3.3	0.3	0.7	0.9	1.5	1.51	2.00	1.32	1.6	Paroxymal hemaglobinuria
65	6.2	2.5	3.7	0.5	0.7	1.0	1.6	1.62	1.95	1.20	1.6	Homologous serum jaundice
66	6.6	3.0	3.6	0.4	0.7	1.0	1.6	1.81	2.14	1.17	1.6	Lupus erythematosus
67	7.9	4.3	3.7	0.3	0.6	0.7	2.1	2.00	2.37	1.18	1.3	Hemolytic jaundice
Mean	7.0	3.6	3.5	0.4	0.6	1.0	1.6	1.64	2.12	1.30	1.6	
68	11.2	2.7	8.5	0.5	0.8	0.8	6.4	6.70	6.45	0.97	1.1	Multiple myeloma
Increased alpha + gamma globulins												
69	7.6	3.3	4.3	0.4	0.9	1.1	1.8	1.87	2.35	1.25	1.6	Rheumatoid arthritis
70	7.5	3.2	4.3	0.6	1.0	1.1	1.7	1.57	1.84	1.17	1.6	Pregnancy 9 months
Increased beta + gamma globulins												
71	7.4	2.4	5.0	0.5	0.6	1.3	2.6	2.77	3.09	1.11	1.5	Infectious hepatitis with jaundice
Increased gamma and decreased beta globulins												
72	6.3	1.6	4.7	0.3	0.6	0.5	3.3	3.03	3.47	1.15	1.2	Multiple myeloma
73	—	—	—	—	—	—	1.4	1.44	1.60	1.11	—	Same case after remission
Plasma sample containing fibrinogen												
74	7.8	3.9	3.9	0.5	0.8	1.0	1.0	1.62	2.23	1.37	1.6 (plus fibrinogen)	Possible pseudohemophilia
				(Fibrinogen = 0.6 g. %)								

TABLE V

Summary of data for flocculation-ninhydrin method for normal and disease cases and comparison with electrophoretic data

Kind of sera used	Electrophoretic data				Flocculation-ninhydrin procedure					
	Gamma glob. *g.* %		Beta+gamma / Gamma		Gamma glob. clot *g.* %		Gamma glob. tot. serum *g.* %		Total serum / Glob. clot	
	Mean, S.D.,	Range	Mean, S.D.,	Range	Mean, S D.,	Range	Mean, S.D.,	Range	Mean, S.D.,	Range
*Normal sera** (80E; 32F-N)	1.0±.21	0.8–1.2	1.9	1.5–2.2	1.00±.13	.72–1.25	1.54±.23	1.09–2.00	1.55±.12	1.40–1.83
Disease sera Increased alpha glob. (8 cases)	1.0±.06	.9–1.1	2.1±.13	1.9–2.3	1.11±.08	.98–1.27	1.70±.10	1.59–1.87	1.55±.12	1.45–1.79
Increased beta glob. (4 cases)	1.0±.10	.9–1.1	2.6±.39	2.2–3.2	1.02±.14	.92–1.26	1.59±.16	1.44–1.85	1.57±.11	1.47–1.73
Increased gamma glob. (8 cases)	1.6±.25	1.3–2.1	1.6±.18	1.3–1.9	1.64±.21	1.32–2.00	2.12±.17	1.93–2.38	1.30±.11	1.17–1.46
Tuberculosis sera† Minimal cases (22E; 4F-N)	1.25±.29	.73–1.94	1.8		1.35±.17	1.07–1.50	2.49±.39	1.94–3.06	1.85±.12	1.70–2.04
Mod. adv. cases (16E; 10F-N)	1.59±.56	.98–2.87	1.7		1.69±.31	1.18–2.42	2.30±.46	1.77–3.56	1.36±.19	1.03–1.74
Far adv. cases (21E; 8F-N)	1.81±.61	.96–3.27	1.7		1.70±.28	1.07–2.23	2.33±.56	1.59–3.45	1.38±.14	1.06–1.55

* Electrophoretic data for 80 normal sera taken from paper by Reiner, Fenichel and Stern (8).
† Electrophoretic data for 59 tuberculosis sera taken from paper by Seibert, Pfaff and Seibert (9).
E = Electrophoretic determinations.
F-N = Flocculation-ninhydrin determinations.

disease cases. The total serum-gamma globulin values are presumed to be equivalent to the electrophoretic gamma globulin plus a beta fraction which corresponds to about 50% of the normal beta globulins. This beta fraction is believed to be composed mainly of $beta_2$ globulins and beta lipoproteins. Experimental evidence to support this viewpoint is furnished by the data obtained with Cohn's fractions in Part I of these papers. These interfering fractions, *i.e.*, III and III–0, have been characterized electrophoretically by Cohn and his associates (10) as containing increased amounts of $beta_2$ globulins and beta lipoproteins. Further experimental support is furnished by the results obtained with lipoid nephrosis sera which have been found by numerous investigators (5, 11–14) to be markedly increased in beta globulins and beta lipoproteins as given in Table III. If these assumptions are correct then changes in the total serum/globulin clot ratio should roughly approximate those in the electrophoretic (beta + gamma/gamma) ratio. The values included in the various tables show this to be true except for some cases of increased beta globulins which will be discussed later.

Campbell and Hanna (15) have shown that 20% sodium sulfite is approximately equal to 20% sodium sulfate for the precipitation of globulins. This concentration has been shown by Majoor (4) to precipitate all the gamma globulin with minimum contamination from other globulins for both normal and disease sera. It is this prior concentration of the gamma globulin by means of salt fractionation that permits the *quantitative* flocculation of gamma globulin from solution over a wide range of concentrations. That this is not possible with salt fractionation alone is shown by the electrophoretic studies of Majoor (4) and Petermann, Young, and Hogness (16) with salt fractionation of both normal and disease sera.

It is clear from the data in this paper that, while salt fractionation followed by the flocculation-ninhydrin procedure effectively separates the gamma globulin fraction from albumin and alpha and beta globulins, no such separation occurs with fibrinogen as can be seen from the plasma run in Table IV, Case No. 74. Since the electrophoretic mobility of fibrinogen lies between the beta and gamma globulins, its interference with the gamma globulin procedure is not unexpected.

From an analytical viewpoint the accuracy of the electrophoretic method is bound to be low because

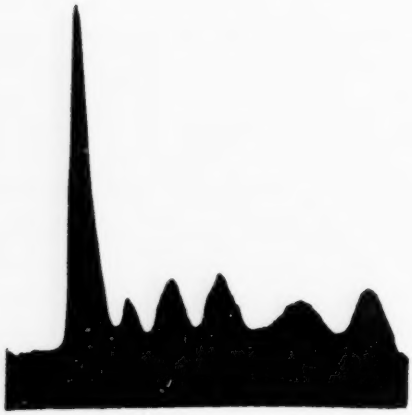

FIG. 1. ELECTROPHORETIC PATTERN FOR NORMAL SERUM

only a single determination is performed on each sample and all subsequent measurements and calculations are based on this *one* determination. Moore and his coworkers (17) have shown that variations in buffer used, hemolysis, and method of dialysis and in period of storage and transportation of sample cause alterations in the electrophoretic pattern obtained. Even measurement of the *same* pattern by different individuals may introduce considerable errors in the final result. When absolute rather than relative concentrations are desired, the error in the micro-Kjeldahl determination may be additive with those of the electrophoretic method. For these reasons, in spite of the much smaller amounts of serum used in the photometric method, *e.g.*, 0.01 to 0.02 ml., the variability of results as measured in a large series of normal and of tuberculosis sera is about one-half that obtained with the electrophoretic method as is shown in Table V. In addition, the data in Table I show the excellent reproducibility of the method and that gamma globulin added to sera may be recovered with an average value of + 105% of the protein added. Repeat values on the same patients' sera, as seen in Tables II and III, gave highly reproducible results with the photometric procedure.

The flocculation-ninhydrin method has other advantages over the electrophoretic method in that it requires no equipment not presently available in most clinical laboratories and in that as many as 100 determinations may be performed each day by one technician as compared to about four per day for the electrophoretic method. This makes the method especially suitable for clinical research involving large numbers of determinations. It is presently being employed for a mass survey of tuberculosis patients, part of the data from such a study being shown in Table V.

The experimental data obtained for human sera in the comparative studies with the electrophoretic and flocculation-ninhydrin methods have been divided into normal cases (Table I) and into disease cases (Tables II–IV) based upon the increase in a particular electrophoretic component. Since extensive data have been compiled for normal sera with the electrophoretic method by numerous investigators as summarized in the paper by Reiner, Fenichel, and Stern (8) there seemed little need to repeat such a study. A small series of normals was therefore run with both procedures and a large series (32 cases) with the flocculation-ninhydrin method alone. Figure 1 shows a typical electrophoretic pattern for a normal serum. A summary of the data obtained for these normal cases is given in Table I and the results compared statistically with the electrophoretic method in Table V.

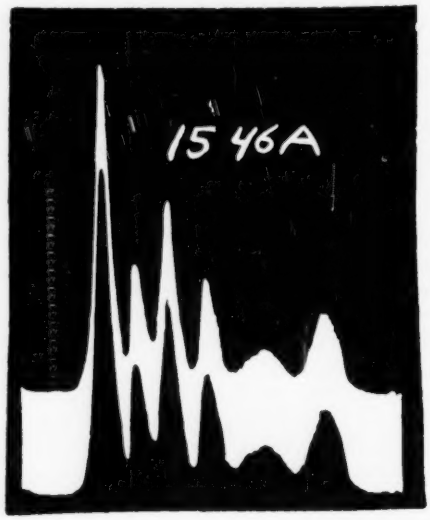

FIG. 2. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF RETICULOCELL SARCOMA (INCREASED ALPHA GLOBULINS)

The method of classification for disease sera based on an increase in a particular electrophoretic component serves to emphasize their relationship with the data obtained with Cohn's fractions in the preceding paper.

The data in Table II lists those cases (Nos. 34 through 41) in which there is an increase in either the alpha$_1$ or alpha$_2$ globulin component. The average increase in each case is about twice that for normal sera. These sera correspond to the runs in Part I dealing with Fractions IV–1 and IV–4 which were non-interfering fractions. Table II shows the excellent agreement between the electrophoretic gamma globulin values and the flocculation-ninhydrin values. The mean value of the total serum/globulin clot ratio for this series of cases is 1.55 as compared to a mean value of 1.55 for normal sera. The electrophoretic (beta + gamma/gamma) ratio is 2.1 for this series as compared to a mean value for normal sera of 1.9. The fact that increases in the alpha globulins have no effect on the flocculation-ninhydrin method for gamma globulin is shown by the data for Case Nos. 42, 43, and 44 where an increase in both alpha and gamma globulin causes a decrease in the ratio *below* normal values, whereas increased alpha globulin and decreased gamma globulin cause a rise in the ratio above normal values. These are the results that would be expected from changes in the gamma globulin alone.

In disease cases reversal of the electrophoretic (albumin/globulin) ratio is of rather frequent occurrence. Because experimental work with the albumin fraction in Part I has shown it to be a non-interfering fraction for the gamma globulin method, changes in this fraction were ignored in classifying the electrophoretic data in this paper. However, in certain cases, *e.g.,* reticulocell sarcoma (Nos. 34, 35, and 36), there is such a marked reversal of the A/G ratio, that such changes can be readily detected with the more insensitive salt fractionation methods. Therefore, where a patient's serum has a normal gamma globulin value, a normal total serum/globulin clot ratio (indicating a normal or slightly increased beta component) and a marked increase in the total globulin over the albumin component (as determined by the usual chemical methods for the A/G ratio), it can be generally assumed that there is an increase in the alpha globulin content of the patient's serum.

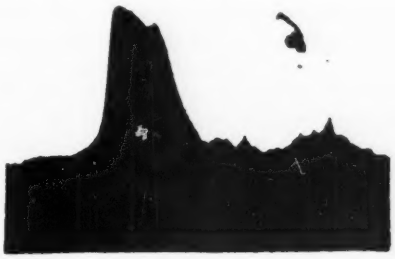

FIG. 3. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF LIPOID NEPHROSIS (INCREASED BETA GLOBULINS AND BETA LIPOPROTEINS)

The flocculation-ninhydrin procedure described in this paper can therefore be used in conjunction with A/G ratio determinations as a gross measure of increases in the electrophoretic alpha globulin values. Figure 2 shows the electrophoretic pattern obtained for a case of reticulocell sarcoma.

The data for the effect of increased beta globulins on the flocculation-ninhydrin method for gamma globulin, as given in Table III, show that the values obtained for gamma globulin with the two procedures are in excellent agreement. This furnishes experimental proof that the interfering beta globulin fractions described in Part I are effectively removed during the procedure. From the increase in the electrophoretic (beta + gamma/gamma ratio), a rise in the total serum/globulin clot ratio would be expected. However, the mean value of this ratio for Nos. 49 through 52 is only 1.57 as compared to a value of 1.55 for normal sera. It is quite possible that the rise in beta globulins in these cases is due solely to an increase in beta$_1$ globulins which are not flocculated out of the diluted serum by this procedure. Experimental proof of this contention can only be furnished by the use of an instrument which can separate the beta globulin into beta$_1$ and beta$_2$ fractions and which is not presently available to the authors. As would be expected an increase in beta globulin accompanied by an increase in gamma globulin causes a fall in the total serum/globulin clot ratio as can be seen in Nos. 54 through 57. Whereas in cases where the increase in beta globulins is accompanied by a decrease in gamma globulin the total serum/globulin clot ratio rises. The flocculation-ninhydrin method should be especially useful in following the prog-

nosis in cases of lipoid nephrosis, Nos. 59 and 60, where the ratio rises to four times the normal value and where the electrophoretic patterns, as seen in Figure 3, are difficult to evaluate quantitatively.

The data for sera containing increased gamma globulin are given in Table IV. In this series both the mean value of the electrophoretic (beta + gamma/gamma) ratio and the total serum/globulin clot ratio show significant decreases from the corresponding values for normal sera. In fact, as the gamma globulin increases from 1.32 g. % to 2.00 g. %, there is a corresponding continuous decrease in the ratio from 1.46 to 1.18. This decrease in the ratio is especially marked in cases of multiple myeloma. A typical electrophoretic pattern obtained with a multiple myeloma serum is shown in Figure 4. It is interesting to note that all three liver disease cases (Nos. 65, 67, and 71) fall into the same series and that all three have ratios below 1.20. Figure 5 shows an electrophoretic pattern for a case of homologous serum jaundice.

In view of the large number of semi-quantitative protein flocculation tests which have appeared in the literature, it should perhaps be emphasized that it is not the intention of the authors to add another such flocculation test to the medical literature. Rather it is the purpose of the authors to conduct investigations on the physico-chemical concepts upon which all flocculation reactions are based and to employ these basic concepts in conjunction with quantitative reactions for the direct quantitative microdetermination of various protein fractions which may or may not correspond to those obtained electrophoretically. Work is presently in progress on the application of these quantitative flocculation methods to the determination of fibrinogen and albumin in plasma, and on the application of these methods to other biological fluids, *e.g.*, cerebrospinal fluid.

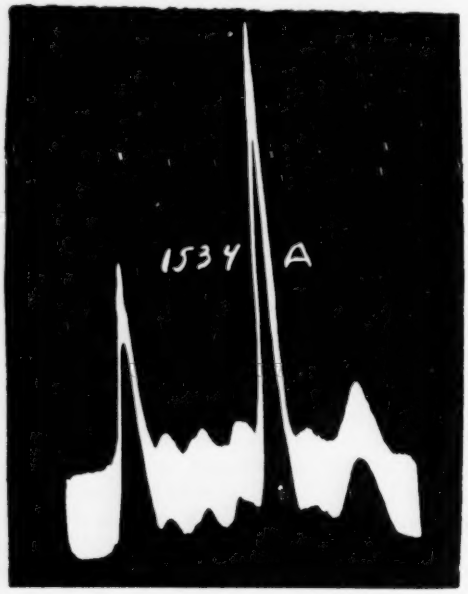

FIG. 4. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF MULTIPLE MYELOMA (INCREASED GAMMA GLOBULIN)

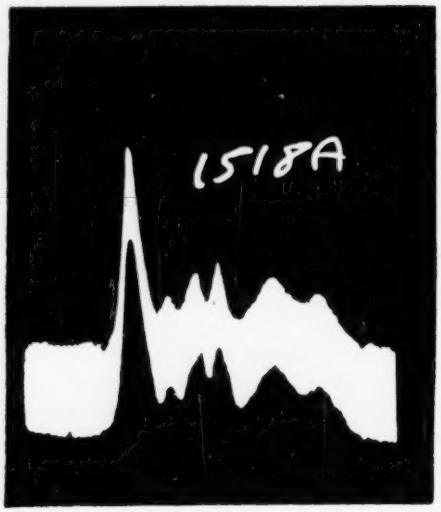

FIG. 5. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF HOMOLOGOUS SERUM JAUNDICE (INCREASED GAMMA GLOBULIN)

SUMMARY

1. The application to human sera of a previously described photometric flocculation-ninhydrin method for the quantitative microdetermination of gamma globulin is described.

2. The principle of the method is based upon precipitation of gamma globulin quantitatively, with a minimum contamination from the other globulin fractions, by means of 20% sodium sul-

fite prior to the application of the flocculation reaction to the dialyzed globulin clot. Results obtained with this procedure are called the globulin clot-gamma globulin values.

3. When the flocculation-ninhydrin method is applied directly to diluted whole sera, the values obtained are usually about 50% higher than the corresponding globulin clot values and are called total serum-gamma globulin values.

4. Fifty sera from both normal and disease cases were run comparatively with the two procedures described above and with the Tiselius electrophoresis apparatus. From these data it was determined that the globulin clot-gamma globulin value corresponds to the electrophoretic gamma globulin value. Also that the total serum-gamma globulin value corresponds to gamma globulin plus another fraction which is believed to be composed of $beta_2$ globulins and beta lipoproteins.

5. Thirty-two normal sera and 25 tuberculosis sera were run with the photometric flocculation-ninhydrin method. The gamma globulin values obtained for each series checked closely with the electrophoretic data reported in the literature by other investigators for a similar series of cases.

6. The total serum/globulin clot ratio was found to be relatively constant for normal sera, *i.e.*, 1.55 $\pm$ .12, and to vary markedly in disease cases. It is not affected by changes in the alpha globulins, *e.g.*, reticulocell sarcoma. It is markedly elevated by increases in beta lipoprotein, especially when accompanied by decreases in the gamma globulin, *e.g.*, lipoid nephrosis. It decreases with increases in the gamma globulin, *e.g.*, multiple myeloma and liver disease, but increases with decreasing gamma globulin, *e.g.*, baby sera.

6. The many advantages of the photometric flocculation-ninhydrin method over the laborious single determination electrophoretic method for clinical work are discussed particularly with respect to the equipment needed and volume of work performed.

7. A statistical analysis of the data obtained with the two procedures shows that the photometric flocculation-ninhydrin method has about one-half the variation of the electrophoretic method in spite of the much smaller sample, *i.e.*, 0.01 to 0.02 ml. of serum used. The method is reproducible to about a $\pm$ 5% error and recovers gamma globulin from sera with an average value of about + 105% of the amount of protein added.

After these papers had been submitted for publication, a paper appeared by Ricketts, Sterling, and Levine (J. Lab. & Clin. Med., 1951, **38**, 153) dealing comparatively with the gamma globulin values obtained by turbidimetric methods (in Part I of these papers, Kunkel [24] and de la Huerga and Popper [8]), which presumably measure gamma globulin quantitatively, and those obtained by the electrophoretic method.

These authors found differences between the turbidimetric and electrophoretic methods of about 20% for normal sera, and as much as 100% for sera from disease cases, *e.g.*, portal or biliary cirrhosis. This furnishes additional experimental evidence to support our viewpoint that such procedures are at best *semi-quantitative* measurements of gamma globulin values.

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REFERENCES

1. Gouy, G., Constitution of the electric charge at the surface of an electrolyte. J. Physique, 1910, (4) **9**, 457.
2. Svensson, H., Fractionation of serum with ammonium sulfate and water dialysis, studied by electrophoresis. J. Biol. Chem., 1941, **139**, 805.
3. Pedersen, K. O., Ultracentrifugal studies on serum and serum fractions. Almquist & Wiksells, Uppsala, 1945.
4. Majoor, C. L. H., The possibility of detecting individual proteins in blood serum by differentiation of solubility curves in concentrated sodium sulfate solutions. II. Comparison of solubility curves with results of electrophoresis experiments. J. Biol. Chem., 1947, **169**, 583.
5. Thorn, G. W., Armstrong, S. H., Jr., Davenport, V. D., Woodruff, L. M., and Tyler, F. H., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXX. The use of salt-poor concentrated human serum albumin solution in the treatment of chronic Bright's disease. J. Clin. Invest., 1945, **24**, 802.

6. Longsworth, L. G., A modification of the schlieren method for use in electrophoretic analysis. J. Am. Chem. Soc., 1939, **61**, 529.
7. Longsworth, L. G., Recent advances in the study of proteins by electrophoresis. Chem. Rev., 1942, **30**, 323.
8. Reiner, M., Fenichel, R. L., and Stern, K. G., Electrophoretic studies on the protein distribution in normal human serum. Acta haemat., 1950, **3**, 202.
9. Seibert, F. B., Pfaff, M. L., and Seibert, M. V., A serum polysaccharide in tuberculosis and carcinoma. Arch. Biochem., 1948, **18**, 279.
10. Cohn, E. J., History of plasma fractionation *in* Advances in Military Medicine. Little, Brown & Co., Boston, 1947, Vol. I, Chap. 28, p. 364.
11. Longsworth, L. G., and MacInnes, D. A., An electrophoretic study of nephrotic sera and urine. J. Exper. Med., 1940, **71**, 77.
12. Luetscher, J. A., Jr., The effect of a single injection of concentrated human serum albumin on circulating proteins and proteinuria in nephrosis. J. Clin. Invest., 1944, **23**, 365.
13. Routh, J. I., Knapp, E. L., and Kobayashi, C. K., Electrophoretic studies of plasma and urinary proteins in children with lipoid nephrosis. J. Pediat., 1948, **33**, 688.
14. Pearsall, H. R., and Chanutin, A., II. Electrophoretic, nitrogen and lipide analyses of plasma and plasma fractions in disease. Am. J. Med., 1949, **7**, 301.
15. Campbell, W. R., and Hanna, M. I., The albumins, globulins and fibrinogen of serum and plasma. J. Biol. Chem., 1937, **119**, 15.
16. Petermann, M. L., Young, N. F., and Hogness, K. R., A comparison of the Howe and the electrophoretic methods for the determination of plasma albumin. J. Biol. Chem., 1947, **169**, 379.
17. Moore, D. H., Roberts, J. B., Costello, M., and Schonberger, T. W., Factors influencing the electrophoretic analysis of human serum. J. Biol. Chem., 1949, **180**, 1147.

THE ULTRAFILTRABILITY OF POTASSIUM AND SODIUM IN HUMAN SERUM

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One of the puzzling clinical aspects of the metabolism of electrolytes is the genesis of symptoms and signs of potassium depletion and potassium intoxication (1–3). Precipitous flaccid paralysis, sensory disturbances and respiratory failure, altogether analogous to the sequence of events in familial periodic paralysis, may occur in these disturbances of potassium metabolism characterized by either deficit or plethora of the ion. The preponderance of evidence indicates that such gross clinical derangements are observed in but a minority of instances of abnormality in the metabolism of potassium (1–9). Review of existing data, furthermore, fails to disclose a clinical, chemical, or physiologic difference which could account for the capricious incidence of serious manifestations in this minority of patients.

The extensive metabolic studies of Jantz (10) in nine cases of familial periodic paralysis seem to afford a clue to the solution of this problem. He found that episodes of paralysis could be consistently related to a profound reduction in the ultrafiltrable fraction of plasma potassium. Although this finding has not been confirmed or negated, such an explanation might in part account for paralytic phenomena which occasionally supervene in patients without familial periodic paralysis, who develop disturbances of potassium metabolism.

The present investigation comprises an analysis of the diffusibility *in vitro* of potassium and sodium of sera obtained from a group of normal subjects and an analogous preliminary survey of patients with derangements of electrolyte metabolism.

METHODS AND MATERIALS

The normal subjects were 24 medical students of the freshman class of Boston University School of Medicine. Patients in various states of electrolyte imbalance and dehydration were studied from the wards of Boston City Hospital. Normal subjects came to the laboratory following an overnight fast. Blood was withdrawn, using minimal stasis, under mineral oil, and serum was separated anaerobically within one hour of clot formation. A portion of serum from the centrifuged blood was aspirated from beneath its thin protective coat of oil into Lavietes' ultrafiltration chambers[3] (11). Anaerobic ultrafiltration transpired through cellophane membranes at room temperature for about 48 hours. Technical details and equipment were identical with those described by Lavietes (11). Both original serum and ultrafiltrate were analyzed simultaneously. Serum residue was not analyzed.

Sodium and potassium were determined on the Barclay flame photometer using lithium as the internal standard (12). All fluids were analyzed in duplicate—*i.e.*, two separate dilutions of 1 to 50 and 1 to 100 for potassium and 1 to 200 and 1 to 400 for sodium. Each unknown solution was "bracketed" by two knowns, and serum and corresponding ultrafiltrate were read together. Final values were averages of the results of two or more readings of each unknown solution. Average readings of duplicate dilutions were within 2% of one another. Before each group of determinations, the accuracy and precision of the instrument were further verified by analyzing samples of "artificial serum"—a carefully compounded known solution of salts of potassium, sodium, calcium, and magnesium in such proportion that concentrations of these cations were within the range of normal human blood serum.

Water of serum and ultrafiltrate was estimated by the method of Eisenmann, Mackenzie, and Peters (13). In a minority of instances insufficient quantities of ultrafiltrate were available, so that the water content was assumed to be that of the arithmetic mean of the series of ultrafiltrates actually analyzed. Distribution ratios of potassium and sodium are expressed as quotients of their concentrations in water of ultrafiltrate divided by concentration in water of serum.

Conventional methods of statistical analyses, as described by Mainland (14), were utilized.

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[2] Part of the data upon which this report is based is taken from a thesis submitted to the Graduate School of Boston University by E. S. H. in partial fulfillment of the requirements for the degree of Master of Arts.

[3] Obtained through the courtesy of F. Pierce Noble, 110 Benham Street, New Haven, Connecticut.

RESULTS

Chemical data and diagnoses are presented in Tables I and II.

The ranges of serum potassium and sodium values in the group of 24 normal subjects are 3.82 to 4.49 meq./l. and 139.8 to 148.9 meq./l., respectively (Table I). The means were 4.16 ± 0.22 meq./l.[4] for potassium and 142.5 ± 2.0 meq./l. for sodium. With respect to the upper limit of the sodium range, it should be noted that only one result exceeded 144.8; that value (148.9) deviated from the mean by more than three standard deviations and may therefore be considered anomalous, at least from the statistical standpoint.

The average distribution ratios of electrolytes in water of ultrafiltrate and serum, in 16 normal subjects (Table I), are 0.943 ± 0.009 for potassium and 0.942 ± 0.007 for sodium. Their ranges were 0.926 to 0.957, and 0.929 to 0.954, respectively. The ultrafiltrability of sodium and of potassium under the conditions of these experiments was therefore nearly identical.

Distribution ratios during 19 evaluations in a series of ten patients (Table II) in whom concentrations of serum potassium and sodium varied tremendously were strikingly similar to those observed in the control group; the patients displayed either potential or definite and flagrant disturbances of electrolyte and water metabolism. Mean ratios of 0.939 ± 0.043 in the case of potassium and 0.944 ± 0.046 for sodium were almost indistinguishable from their counterparts in normal subjects. The ranges of 0.909 to 0.960 (K) and 0.920 to 0.966 (Na) were, however, wider than the corresponding values in the group of normal subjects. A greater degree of variability is likewise evident as defined by the larger standard deviations. It appears clear, nevertheless, that the diffusibility of both potassium and sodium in these patients did not differ significantly from that of the normal group.

[4] Unless otherwise stated, the plus or minus figure following the mean refers to standard deviation.

TABLE I

The distribution of sodium and potassium between serum and in vitro *ultrafiltrate of serum in normal subjects*

Subj., age	Serum		Ultrafilt.		Water		(K)u*	(Na)u*	(K)s*	(Na)s*	(K)u/(K)s	(Na)u/(Na)s
	K	Na	K	Na	Ser.	Ult.						
	meq./l.	*meq./l.*	*meq./l.*	*meq./l.*	*gms./l.*	*gms./l.*	*meq./l.*	*meq./l.*	*meq./l.*	*meq./l.*		
H. C. 25	3.93	142.2	4.01	142.2	933		4.03	143.0	4.21	152.4	0.957	0.938
C. F. 24	4.35	140.9	4.39	142.7	937		4.42	143.5	4.64	150.4	0.953	0.954
H. S. 21	4.49	142.3	4.45	141.9	930		4.48	142.7	4.83	153.2	0.928	0.931
A. B. 20	4.18	142.7	4.13	143.7	933		4.15	144.5	4.48	152.9	0.926	0.945
M. H. 24	4.43	148.9	4.48	148.5	926		4.51	149.4	4.79	160.9	0.942	0.929
J. L. 26	4.27	142.8	4.28	143.5	933	996	4.30	144.1	4.58	153.0	0.939	0.942
J. M. 24	4.11	139.8	4.13	140.8	928	994	4.16	141.7	4.43	150.6	0.939	0.941
H. W. 21	4.04	142.0	4.03	143.2	932	992	4.06	144.4	4.33	152.3	0.938	0.948
M. B. 22	4.25	144.8	4.32	145.6	933	996	4.34	146.1	4.55	155.1	0.954	0.942
R. O. 30	3.90	144.7	3.87	145.7	936		3.89	146.5	4.17	154.6	0.933	0.948
M. B. 22	4.35	142.4	4.38	142.4	931	994	4.41	143.2	4.67	153.0	0.944	0.936
D. W. 27	4.06	142.9	4.12	143.1	931	993	4.15	144.0	4.36	153.5	0.952	0.938
N. W. 21	3.92	143.4	3.93	144.6	932	995	3.95	145.3	4.21	153.8	0.938	0.945
C. P. 29	4.39	141.8	4.43	142.9	930	992	4.46	144.0	4.72	152.4	0.945	0.945
T. L. 27	4.14	142.9	4.19	144.9	932	994	4.22	145.7	4.44	153.4	0.950	0.950
A. K. 25	4.11	140.1	4.14	140.5	936	996	4.16	141.0	4.39	149.7	0.948	0.942
M. D. 24	4.49	144.0			927				4.84	155.3		
Z. L. 24	4.37	142.3			929				4.71	153.3		
G. H. 22	3.82	140.2			935				4.09	149.9		
A. R. 24	3.82	142.8			928				4.12	153.8		
S. F. 26	4.11	139.8			932				4.41	150.0		
H. L. 24	3.91	143.1			927				4.22	154.4		
P. L. 25	4.45	141.7			931				4.78	152.2		
M. A. 29	4.06	140.3			930				4.37	150.9		
Mean	4.16	142.5									0.943	0.942
St. dev.	0.22	2.0									0.009	0.007
St. error	0.05	0.4									0.002	0.002

* These concentrations of potassium and sodium have been corrected for water content of ultrafiltrate and of serum by using analytical data or, in the case of certain ultrafiltrates, mean values of other analyses (*cf.* Methods).

TABLE II

The distribution of sodium and potassium between serum and in vitro *ultrafiltrate of serum in patients with disturbances of electrolyte metabolism*

Pt.	Remarks	Serum		Ultrafilt.		Water		(K)u*	(Na)u*	(K)s*	(Na)s*	$\frac{(K)u}{(K)s}$	$\frac{(Na)u}{(Na)s}$
		K	Na	K	Na	Ser.	Ult.						
		meq./l.	*meq./l.*	*meq./l.*	*meq./l.*	*gms./l.*	*gms./l.*	*meq./l.*	*meq./l.*	*meq./l.*	*meq./l.*		
B.	Renal insufficiency	4.51	144.4	4.40	143.6	941	994	4.43	144.5	4.79	153.5	0.925	0.941
T.	Diabetes mellitus	4.27	144.1	4.25	144.8	927	975	4.36	148.5	4.61	155.4	0.946	0.956
H.	Diabetes mellitus	6.20	141.6	6.20	141.2	930		6.27	142.8	6.67	152.3	0.940	0.938
S.	Myocardial infarction	5.69		5.69		934		5.75		6.09		0.944	
T.	Diabetic acidosis	5.87	144.2	6.04	144.9	904	987	6.12	146.8	6.50	159.5	0.942	0.920
		3.20	143.8	3.19	146.2	923	987	3.23	148.1	3.47	155.8	0.931	0.951
R.	Renal insufficiency	3.25	123.9	3.25	120.6	936	999	3.25	120.7	3.47	132.4	0.936	0.911
		3.16	130.1	3.07	129.0	934	993	3.09	129.9	3.38	139.3	0.914	0.932
K.	Renal and hepatic insufficiency	4.46	131.5	4.32	129.3	950	989	4.37	130.7	4.69	138.4	0.931	0.944
		4.07	132.2	4.04	132.0	945	989	4.08	133.5	4.31	139.9	0.946	0.954
		4.51	132.2	4.52	131.1	942	988	4.57	132.7	4.79	140.3	0.954	0.945
S.	Diabetic acidosis	4.91	124.5	5.07	129.5	919		5.13	130.9	5.34	135.5	0.960	0.966
		2.37	136.3	2.35	135.9	946		2.38	137.4	2.51	144.1	0.948	0.954
		2.50	131.5	2.50	132.5	948		2.53	134.0	2.64	138.7	0.958	0.966
M.	Intestinal obstruction	2.89	135.4	2.80	135.1	930		2.83	136.6	3.11	145.6	0.909	0.938
L.	Renal insufficiency	7.75	117.3	7.80	118.3	929		7.89	119.6	8.34	126.3	0.946	0.947
		7.92	117.1	7.99	117.1	921		8.08	118.4	8.60	127.1	0.939	0.932
		8.47	120.1	8.37	122.0	933		8.46	123.4	9.08	128.7	0.931	0.959
		9.13	115.6	9.22	116.6	930		9.32	117.9	9.82	124.3	0.949	0.949
Mean												0.939	0.944
St. dev.												0.043	0.046
St. error												0.010	0.013

* These concentrations of potassium and sodium have been corrected for water content of ultrafiltrate and of serum by using analytical data or, in the case of certain ultrafiltrates, mean values of other analyses (*cf.* Methods).

DISCUSSION

Concentrations of serum potassium and sodium in the present series of normal subjects are in reasonable agreement with certain previously reported series (12, 15). Distribution ratios of potassium and sodium in serum of these subjects corresponded very closely to predictions of the Gibbs-Donnan equilibrium (16). This ratio in the case of sodium coincides almost exactly with mean values already reported in man and other animals (17–23). The distribution coefficient of potassium approximated that found in work on dogs (20) and in recent studies of Folk, Zierler, and Lilienthal (23) as well as of Berliner, Kennedy, and Hilton (24). Such distribution ratios of sodium and potassium confirm the assumption usually made with respect to concentrations of the ions in extracellular fluid in relation to certain balance calculations (4, 25).

Distribution ratios of potassium ascertained by earlier investigators were, in the vast majority of instances, below 0.90. Although the explanation for this disparity is unclear, certain of the following factors may well have been involved: unreliability of earlier analytical methods for determination of potassium; failure to adhere to the excellent methodologic criteria and precautions pertaining to ultrafiltration studies outlined by Ingraham, Lombard, and Visscher and implemented by Lavietes (21, 11); differences in technique of ultrafiltration including study of *in vivo*, rather than *in vitro*, transudates; species differences. Finally, the range of variation of the distribution ratios of both sodium and potassium in the present group of normal individuals is considerably less than has been found by previous investigators.

No specific or definite symptoms or signs which could be clearly ascribed to abnormalities in potassium metabolism were observed in these patients. Whether paralysis and related disturbances occur only when abnormal binding of potassium, presumably to protein, supervenes cannot therefore be gleaned from the present study. Search for suitable patients with neuromuscular abnormalities definitely attributable to abnormal metabolism of potassium continues. Although the occasional occurrence of this correlation appears to be incon-

trovertible, its rarity is documented by the fact that we have not observed it during the observation and study, over several years, of many patients with potassium deficiency and intoxication.

SUMMARY

1. The diffusibility of serum potassium and sodium was measured *in vitro*, using Lavietes' technique, in a group of normal subjects and in a series of patients with latent or striking disturbances in the metabolism of electrolytes.

2. The mean concentrations of serum sodium and potassium in 24 medical students were 142.5 ± 2.0 meq./l. and 4.16 ± 0.22 meq./l., respectively, The average distribution ratios of potassium and sodium expressed as the quotient of concentration in water of ultrafiltrate divided by concentration in water of serum were 0.943 ± 0.009 and 0.942 ± 0.007.

3. Despite impressive differences in total concentration of potassium and sodium in 16 studies of ten patients, their ultrafiltrable fractions did not appear to differ significantly from those of the normal series.

4. Implications of these findings for certain clinical aspects of the metabolism of potassium are discussed.

REFERENCES

1. Finch, C. A., Sawyer, C. G., and Flynn, J. M., The clinical syndrome of potassium intoxication. Am. J. Med., 1946, **1**, 337.
2. Danowski, T. S., Newer concepts of the role of potassium in disease. Am. J. Med., 1949, **7**, 525.
3. Elkinton, J. R., and Tarail, R., The present status of potassium therapy. Am. J. Med., 1950, **9**, 200.
4. Darrow, D. C., The retention of electrolyte during recovery from severe dehydration due to diarrhea. J. Pediat., 1946, **28**, 515.
5. Tarail, R., and Elkinton, J. R., Potassium deficiency and the role of the kidney in its production. J. Clin. Invest., 1949, **28**, 99.
6. Greenman, L., Mateer, F. M., Gow, R. C., Peters, J. H., and Danowski, T. S., Some observations on the development of hypokaliemia during therapy of diabetic acidosis in juvenile and young adult subjects. J. Clin. Invest., 1949, **28**, 409.
7. Mateer, F. M., Greenman, L., Austin, A. C., Peters, J. H., Mermelstein, H., Weigand, F., and Danowski, T. S., The use of potassium chloride in the preoperative treatment of pyloric stenosis in infants. Am. J. M. Sc., 1951, **221**, 21.
8. Elkinton, J. R., Tarail, R., and Peters, J. P., Transfers of potassium in renal insufficiency. J. Clin. Invest., 1949, **28**, 378.
9. Merrill, J. P., Levine, H. D., Somerville, W., and Smith, S., Clinical recognition and treatment of acute potassium intoxication. Ann. Int. Med., 1950, **33**, 797.
10. Jantz, H., Stoffwechseluntersuchungen bei paroxysmaler Lähmung. Nervenarzt, 1947, **18**, 360.
11. Lavietes, P. H., Anaerobic ultrafiltration. J. Biol. Chem., 1937, **120**, 267.
12. Wallace, W. M., Holliday, M., Cushman, M., and Elkinton, J. R., The application of the internal standard flame photometer to the analysis of biologic material. J. Lab. & Clin. Med., 1951, **37**, 621.
13. Eisenmann, A. J., Mackenzie, L. B., and Peters, J. P., Protein and water of serum and cells of human blood, with a note on the measurement of red blood cell volume. J. Biol. Chem., 1936, **116**, 33.
14. Mainland, D., The Treatment of Clinical and Laboratory Data. Oliver & Boyd, Edinburgh, 1938.
15. Hald, P. M., Notes on the determination and distribution of sodium and potassium in cells and serum of normal human blood. J. Biol. Chem., 1946, **163**, 429.
16. Peters, J. P., Body Water: The Exchange of Fluids in Man. Charles C Thomas, Springfield, Ill., 1935.
17. Loeb, R. F., Atchley, D. W., and Palmer, W .W., On the equilibrium condition between blood serum and serous cavity fluids. J. Gen. Physiol., 1922, **4**, 591.
18. Hastings, A. B., Salveson, H. A., Sendroy, J., and Van Slyke, D. D., Studies of gas and electrolyte equilibria in blood; distribution of electrolytes between transudates and serum. J. Gen. Physiol., 1927, **8**, 701.
19. Greene, C. H., and Power, M. H., The distribution of electrolytes between serum and the in vivo dialysate. J. Biol. Chem., 1931, **91**, 183.
20. Greene, C. H., Bollman, J. L., Keith, N. M., and Wakefield, E. G., The distribution of electrolytes between serum and transudates. J. Biol. Chem., 1931, **91**, 203.
21. Ingraham, R. C., Lombard, C., and Visscher, M. B., The characteristics of ultrafiltrates of plasma. J. Gen. Physiol., 1933, **16**, 637.
22. Gilligan, D. R., Volk, M. C., and Blumgart, H. L., Observations on the chemical and physical relation between blood serum and other body fluids. I. The nature of edema fluids and evidence regarding the mechanism of edema formation. J. Clin. Invest., 1934, **13**, 365.
23. Folk, B. P., Zierler, K. L., and Lilienthal, J. L., Jr., Distribution of potassium and sodium between serum and certain extracellular fluids in man. Am. J. Physiol., 1948, **153**, 381.
24. Berliner, R. W., Kennedy, T. J., Jr., and Hilton, J. G., Renal mechanisms for the excretion of potassium. Am. J. Physiol., 1950, **162**, 348.
25. Elkinton, J. R., and Winkler, A. W., Transfers of intracellular potassium in experimental dehydration. J. Clin. Invest., 1944, **23**, 93.

STUDIES ON THE CONCENTRATION AND BACTERIAL EFFECT OF AUREOMYCIN IN DIFFERENT PORTIONS OF THE INTESTINAL TRACT

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Numerous investigations have been carried out dealing with the absorption of aureomycin into the blood stream, body fluids, and tissues (1, 2). However, no determination of the concentration of this antibiotic within the lumen of the intestine has been reported. Likewise, there have been many studies concerning the effect of oral aureomycin on the intestinal bacterial flora, but these have relied upon the examination of stool specimens (3). It was believed that a determination of the concentration of aureomycin and its bacterial effect in various portions of the intestine would be of value in establishing more accurately the role of this antibiotic in intestinal disease.

For these reasons, it was decided to investigate patients having a cecostomy, a transverse colostomy or a sigmoid colostomy. In this manner, it was possible to correlate oral dosage and intestinal aureomycin levels as well as alterations in the bacterial flora in different portions of the intestinal tract. Similar studies were carried out using stool specimens from a group of hospital patients having no apparent intestinal disease. It was recognized that a comprehensive investigation of intestinal bacteria involving aerobic and anaerobic studies would be an extensive project. A review of the bacteriologic records of the John Gaston Hospital for the five year period from January 1, 1945 to January 1, 1950 disclosed that eight pathogenic organisms were most frequently isolated in cases of peritonitis resulting from perforation of the gastrointestinal tract: *Escherichia coli, Streptococcus anaerobius, Bacteroides* organisms, *Streptococcus pyogenes, Streptococcus viridans, Streptococcus faecalis, Clostridium perfringens,* and *Aerobacter aerogenes.* It was decided to restrict the bacteriologic studies to these eight organisms.

METHODS

Clinical methods. Nine patients having a cecostomy, a transverse colostomy or a sigmoid colostomy were studied. Two of these patients had a cecostomy; three had a transverse colostomy; and four had a sigmoid colostomy. The following procedure was carried out in every case: after obtaining two control specimens, each patient was given 250 mg. of aureomycin orally at 10:00 a.m., 2:00 p.m., 6:00 p.m., and 10:00 p.m. for two days; then, four days after the intestinal or fecal aureomycin had disappeared, this procedure was repeated using 500 mg. instead of 250 mg. of aureomycin; again, after the aureomycin level in the intestine or feces had been nil for four days, 750 mg. of aureomycin were administered four times a day for two days. Medication was followed in every instance with 100 cc. of milk. This particular regimen was adopted because it corresponded to the routine anti-amebic treatment employed at the John Gaston Hospital (4). Intestinal specimens were obtained at approximately the same period every 24 hours. One of the two cecostomy patients was unable to remain in the hospital more than 10 days. Therefore, only the 2 gm. regimen was carried out in that case.

In addition to the nine cecostomy and colostomy cases, 20 hospital patients having no apparent gastro-intestinal disease were investigated following the oral administration of aureomycin as described above. Efforts were made to obtain stool specimens daily for five days in each of these cases. Four of these patients were subjected to the 1 gm. regimen, nine to the 2 gm. regimen and seven to the 3 gm. regimen. Attention was especially directed toward the 2 gm. group because it corresponded to our routine anti-amebic therapy (4).

Assay methods. The method employed for determining aureomycin concentration in feces and intestinal material was suggested by Dornbush (5). Four gm. of intestinal material or feces were placed in a large centrifuge tube; then, 100 cc. of acid acetone were added and, after mixing well, this combination was allowed to stand for 10 minutes. Following this, centrifugation was carried out for 10 minutes at 15,000 r.p.m. Thereafter, 1 cc. of the supernatant was withdrawn and used for serial twofold dilutions in Penassay broth. Twenty tubes were employed for each determination. The first tube contained 0.5 cc. of undiluted supernatant, tubes 2 through 19 contained 0.5 cc. of serial dilutions of the supernatant, and the last tube contained broth alone as a bacterial control.

When a high concentration of aureomycin was suspected, a 1 : 10 dilution of the original extraction material was prepared and 1 cc. of this was treated exactly as was the undiluted material. After carrying out the above procedure, 1.5 cc. of a 1 : 100 dilution of an overnight Penassay broth (Difco) culture of *Bacillus cereus* was added to each tube and the contents were mixed. Following this, the tubes were incubated at 37° C. for four hours and 10 minutes. The additional 10 minutes were allowed for heating the tubes initially to 37° C. This entire process was carried out in duplicate.

After incubation, the tubes were read for the amount of aureomycin as is described below. In every instance, two control series were carried out. The first of these utilized crystalline aureomycin in 2 mcg. per cc. concentration. This portion of the determination was conducted exactly as above except that 1 cc. of Penassay broth (Difco) containing 2 mcg. of aureomycin was employed instead of the extraction material. The aureomycin control usually revealed absence of growth through tube 8, so that inhibition through tube 7 in the assay determination represented 1 mcg. of aureomycin per cc. of the extraction material (or 1 mcg. of aureomycin per 0.04 gm. of intestinal or fecal material). In rare instances, there was a variation of one tube in the aureomycin control determination. The tests were read as follows (for this illustration, it is assumed that inhibition in the aureomycin control series extended through tube 8):

Tube: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Growth: + (1 mcg. aureomycin per 0.04 gm. of intestinal or fecal material; that is, 1 mcg. per cc. of extraction material)
++ (0.5 mcg. of aureomycin per 0.04 gm. of fecal or intestinal material)
+++ (0.25 mcg. of aureomycin per 0.04 gm. of fecal or intestinal material).

The second control was performed exactly as the first except that 1 cc. of acid acetone was employed, instead of 2 mcg. of crystalline aureomycin in 1 cc. of Penassay broth. Inhibition in this control series varied from tubes 1 to 4. Therefore, whenever in the assay of intestinal or fecal material, inhibition was the same in the test series and in the acid acetone control, the aureomycin concentration was read as 0. In early determinations, it was found that no difference in levels occurred when the crystalline aureomycin was dissolved in Penassay broth or acid acetone. The acid acetone employed in extracting aureomycin from fecal or intestinal material was prepared as follows: 1 part of 4 N hydrochloric acid, 6 parts of water and 13 parts of acetone.

In most instances, aureomycin assay determinations were carried out within one hour of the receipt of the intestinal or fecal specimens; in no case was there an interval exceeding two hours. Prior to this procedure the material was maintained at −20° C. Two intestinal or fecal specimens were obtained in each case as controls before the initial aureomycin regimen was instituted. No evidence of aureomycin was noted in any of the pre-treatment control specimens. While no history of the recent use of antibiotics or chemotherapeutic agents could be obtained in any patient selected for this investigation, these control specimens provided additional protection against such a possibility.

Bacteriologic methods. Stool and intestinal specimens were collected at approximately the same period every 24 hours in autoclaved dry containers. Cultures were made within one to two hours after the collection of specimens. The following procedure was employed for this purpose: approximately 1 gm. of feces or intestinal material was transferred aseptically to 10 cc. of sterile saline and thoroughly mixed. One cc. of this material was transferred to a tube containing 9 cc. of sterile physiological saline and the contents again were thoroughly mixed. This procedure was carried out through five tubes with the tubes being numbered 1 through 5 consecutively. Fourteen blood agar plates were used for each specimen. These were divided into two sets of seven plates. In each set, the plates were numbered 1 through 7. Two drops of the contents of tube 2 were transferred aseptically to the first blood agar plate and one drop was similarly placed on the second plate. The material was spread evenly on the first plate with a sterile glass spreader. A second spreader was utilized to distribute the material on plate 2 and dilution was then obtained by using the same spreader for the third plate. This procedure was carried out in duplicate. The same method was employed for inoculating three eosin methylene blue plates (Difco). In a similar manner, two drops of the contents of tube 5 were placed on the fourth blood agar plate and the material was then diluted by using the same spreader for the fifth plate. This procedure was also carried out in duplicate. Tube 1 was heated in a water bath at 80° C. for 15 minutes, after which two drops of its contents were placed on the sixth blood agar plate and were subsequently diluted on the seventh plate by using the same glass spreader. One set of blood agar plates and the three eosin methylene blue plates were incubated aerobically at 37° C. for 24 hours. The remaining set of blood agar plates was incubated anaerobically at 37° C. for 48 hours.

The gross appearance of all bacterial colonies was recorded. The first plate was used to determine the number of each type of colony except when it was overgrown. When the first plate could not be employed, the fourth plate was utilized for this purpose; and the number of colonies counted was multiplied by the dilution factor. The amount of growth was recorded as follows:

Negative—no growth
1 +—from 1 to 15 colonies
2 +—from 16 to 50 colonies
3 +—from 51 to 100 colonies
4 +—more than 100 colonies.

RESULTS

Assay results. Using 1 gm. of oral aureomycin daily for two days, intestinal and fecal concentrations of at least 1,000 mcg. per gm. of material were obtained for varying periods in every case except one. In three instances maximum aureomy-

cin concentrations ranged from 51,200 to 102,400 mcg. per gm. Fecal aureomycin levels in four patients, having no cecostomy or colostomy, receiving 1 gm. daily for two days, varied from 3,200 to 51,200 mcg. per gm. of material.

With 2 gm. of oral aureomycin daily, higher concentrations were obtained in the intestine and stool than in the preceding series. Maximal concentrations ranging from 25,600 to 102,400 mcg. per gm. of intestinal material were obtained in seven of the nine cecostomy and colostomy patients. Stool aureomycin levels reaching 25,000 to 50,000 mcg. per gm. of wet material were obtained in every instance (Table I).

With 3 gm. of oral aureomycin daily for two days, persistently higher concentrations were obtained in the intestine and feces than in either of the two preceding groups. Of importance is the fact that significant aureomycin intestinal levels were rapidly attained and were then maintained for two and three days after medication was discontinued. With the exception of the cecostomy case, every patient in this series disclosed intestinal or fecal concentrations of aureomycin reaching 51,600 to 128,000 mcg. per gm. of wet material. Since only graded quantitative differences were noted in the results obtained with 1 gm., 2 gm. and 3 gm. of aureomycin, the concentrations demonstrated in the 2 gm. series alone are recorded in Table I.

The effect of diarrhea on aureomycin intestinal levels is of obvious practical importance in the treatment of intestinal disease, for example, human amebic dysentery. The rapid decrease in the aureomycin level in patient 3 in the 2 gm. series (Table I) was associated with a painless diarrhea of five to seven liquid stools per day. Additional evidence of the effect of diarrhea in reducing aureomycin intestinal levels was observed in the two cecostomy patients in this series (Table I). Patient 1 had a persistent, moderately severe diarrhea throughout this investigation; while the second patient noted no side effects. Concentrations obtained in the latter case were four to 12.5 times greater than in the first patient.

Bacteriologic results. To promote cohesion in discussion and representation, the bacteriologic studies involving the various portions of the intestine—cecum, transverse colon and sigmoid colon—were grouped together as intestinal effects. When a reduction of one of the eight bacteria studied was observed from 4 + or 3 + to 1 + or 0 (as previously explained), it was regarded as evidence

TABLE I

Aureomycin intestinal and stool levels

		2 Gm. of Oral Aureomycin Daily for 2 Days				
Source of Material	Case No.	Mcg. of Aureomycin per Gm. of Wet Material				
		1 DAY After Treatment Began	2 DAYS After Treatment Began	3 DAYS After Treatment Began	4 DAYS After Treatment Began	5 DAYS After Treatment Began
Cecostomy	1	1600	6400	2000	1600	800
	2	—+	51200	25600	12800	3200
Transverse Colostomy	3	102400	64000	200	6	0
	4	25600	64000	3200	—	—
	5	102400	51200	51200	400	200
Sigmoid Colostomy	6	400	6400	800	—	—
	7	25600	51200	51200	12800	6400
	8	800	3200	25600	51200	12800
	9	51200	102400	102400	—	25
Stool	10	—	51200	51200	64000	51200
	11	50	—	25600	1600	1600
	12	25600	25600	—	1600	—
	13	25600	25600	25	—	—
	14	6400	102400	51200	—	6400
	15	—	64000	1600	800	—
	16	25600	102400	51200	—	—
	17	—	102400	51200	—	6400
	18	—	—	12800	6400	6400

+ Indicates "C" specimens were not obtained.

TABLE II

Bacteriologic effects

2 GM. OF ORAL AUREOMYCIN DAILY FOR 2 DAYS																						
	ALTERATIONS IN INTESTINAL BACTERIAL FLORA											ALTERATIONS IN FECAL BACTERIAL FLORA										
ORGANISMS STUDIED	NO OF CASES	1 DAY AFTER TREATMENT BEGAN		2 DAYS AFTER TREATMENT BEGAN		3 DAYS AFTER TREATMENT BEGAN		4 DAYS AFTER TREATMENT BEGAN		5 DAYS AFTER TREATMENT BEGAN		NO OF CASES	1 DAY AFTER TREATMENT BEGAN		2 DAYS AFTER TREATMENT BEGAN		3 DAYS AFTER TREATMENT BEGAN		4 DAYS AFTER TREATMENT BEGAN		5 DAYS AFTER TREATMENT BEGAN	
		+*	−*	+	−	+	−	+	−	+	−		+	−	+	−	+	−	+	−	+	−
ESCHERICHIA COLI	8	2	6	0	8	0	8	2	6	3	5	15	7	8	5	10	2	13	3	12	4	11
STREPTOCOCCUS ANAEROBIUS	3	0	3	0	3	0	3	0	3	2	1	5	1	4	0	5	0	5	0	5	1	4
BACTEROIDES	5	0	5	0	5	0	5	2	3	4	1	5	0	5	0	5	0	5	0	5	2	3
STREPTOCOCCUS VIRIDANS	3	1	2	0	3	0	3	0	3	2	1	4	1	3	0	4	0	4	0	4	1	3
STREPTOCOCCUS PYOGENES	1	0	1	0	1	0	1	0	1	1	0	2	0	2	0	2	0	2	1	1	1	1
STREPTOCOCCUS FECALIS	6	2	4	3	3	3	3	6	0	6	0	5	3	2	4	1	5	0	5	0	5	0
CLOSTRIDIUM PERFRINGENS	6	0	6	0	6	0	6	1	5	3	3	9	1	8	0	9	0	9	1	8	1	8
AEROBACTER AEROGENES	8	2	6	1	7	2	6	2	6	5	3	6	3	3	1	5	0	6	0	6	4	2

* INDICATES SLIGHT OR NO INHIBITION
\+ INDICATES MARKED OR COMPLETE INHIBITION

of marked or complete inhibition. If reduction was only in the range of 4 + to 3 + or 3 + to 2 + or 2 + to 1 + or 1 + to 0 or if there was no decrease, it was regarded as slight or no inhibition. This method was employed with both intestinal and fecal specimens.

Even such a minimal amount of oral aureomycin as 1 gm. daily for two days, produced a rapid intestinal effect. Seven of the eight bacteria studied were markedly or completely inhibited in 24 to 48 hours and definite reduction of these organisms persisted for two days after this small amount of medication was discontinued. Only *Streptococcus faecalis* was not uniformly responsive.

Using 2 and 3 gm. of aureomycin daily for two days, a more rapid and marked reduction of bacteria occurred. While 1 gm. resulted in the complete inhibition of two of the eight bacteria in the intestine in 24 hours, 2 gm. completely inhibited three of these organisms (Table II), and 3 gm. caused the complete inhibition of six. Similar effects were noted in the fecal specimens. Only *Streptococcus faecalis* was not uniformly reduced to a significant degree. However, when 3 gm. were administered daily for two days, three of five intestinal cases revealed complete or marked inhibition of this organism, though only one of five of the stool specimens disclosed this degree of reduction. In all cases, the effect on *Streptococcus faecalis* was brief. A definite inhibition of the other seven bacteria in the intestine and stool was evident for 48 and 72 hours after aureomycin was discontinued.

DISCUSSION

In an effort to evaluate more exactly the role of aureomycin in intestinal disease, material from various portions of the intestine was examined following the administration of therapeutic amounts of this antibiotic. Each specimen was studied to determine the concentration of aureomycin and its effect on eight pathogenic organisms. Using 2 to 3 gm. of oral aureomycin daily, intestinal concentrations of this agent were obtained which reached 51,200 to 128,000 mcg. per gm. of wet material.

These initial aureomycin intestinal levels are quite suggestive as regards the mode of action of this antibiotic in human amebiasis. In 1949, it was demonstrated that aureomycin is amebicidal *in vitro* (6). Further evidence indicating a direct effect by this antibiotic on *E. histolytica* in the test tube was presented by Hewitt and his associates who described the complete destruction of amebae by aureomycin in concentrations of 1,000 mcg. per cc. with only 10 minutes of contact (7). More recently, Hall reported that aureomycin destroyed *E. histolytica* trophozoites *in vitro* in concentrations of 50 mcg. per cc. (8).

After *in vitro* studies, more than 100 cases of

amebiasis were successfully treated at the John Gaston Hospital with oral aureomycin. However, the exact mode of action of this antibiotic in the body was not clear. There remained the possibility that aureomycin exerted its effect in the body primarily through an indirect action on amebae by altering the intestinal bacterial flora. That this antibiotic has a broad antibacterial spectrum of activity is well established.

The results of the present studies indicate that the oral administration of therapeutic amounts of aureomycin produces intestinal concentrations which are 50 to 100 times greater than established amebicidal levels *in vitro* (51,200 to 128,000 mcg. per gm. of wet material). These first aureomycin intestinal levels suggest, therefore, that this agent exerts its major therapeutic effect in human amebiasis through a direct action on *E. histolytica*. It is generally recognized that the eradication of amebic infections requires the destruction of amebae in tissue as well as in the intestinal lumen. In view of the high concentration of aureomycin obtained within the lumen of the colon, it appears probable that amebicidal amounts penetrate the intestinal wall via the vascular system. It is apparent from the present investigation that while diarrhea causes a reduction in the intestinal concentration of aureomycin, levels are still present which are amebicidal *in vitro*. The alteration of the intestinal bacterial flora may play a part in the destruction of *E. histolytica* in the body, but this would appear to be of secondary importance.

Bacteriologic studies restricted to eight common pathogenic organisms revealed that 2 to 3 gm. of oral aureomycin daily for two days resulted in a rapid and marked inhibition of seven of these bacteria. This effect persisted for 48 to 72 hours after medication was discontinued. *Streptococcus faecalis* alone was not uniformly responsive; though when 3 gm. were administered daily, this organism was markedly reduced in more than half of the cases revealing its presence. While the intestinal antibacterial spectrum of aureomycin has been extensively investigated (3), it is believed that information derived from this study of material from different portions of the intestine may be of value in determining more exactly its role in intestinal surgery. In view of the rapid attainment and subsequent maintenance of significant intestinal levels together with its bacterial effects, it appears that aureomycin should be an excellent agent for the preoperative preparation of the intestine. Clinical reports substantiate this view (9, 10).

SUMMARY

1. The oral administration of therapeutic amounts of aureomycin results in the rapid attainment and rather prolonged maintenance of significant intestinal concentrations of this antibiotic.

2. These first aureomycin intestinal levels indicate that this antibiotic probably exerts its major therapeutic effect in human amebiasis by a direct action on *E. histolytica;* though there may be a minimal indirect action on amebae resulting from alteration of the intestinal bacterial flora.

3. The studies reported in this paper dealing with the concentration of aureomycin and its bacteriologic action in various portions of the intestine indicate that this antibiotic should be an effective agent for preoperative preparation of the intestine.

REFERENCES

1. Dornbush, A. C., and Pelcak, E. J., The determination of aureomycin in serum and other body fluids. Ann. New York Acad. Sc., 1948, **51**, 218.
2. Herrell, W. E., and Heilman, F. R., Aureomycin studies on absorption, diffusion and excretion. Proc. Staff Meet., Mayo Clinic, 1949, **24**, 157.
3. Dearing, W. H., and Heilman, F. R., The effect of aureomycin on the bacterial flora of the intestinal tract of man: a contribution to preoperative preparation. Proc. Staff Meet., Mayo Clinic, 1950, **25**, 87.
4. McVay, L. V., Jr., Laird, R. L., and Sprunt, D. H., The treatment of amebiasis with aureomycin. South. M. J., 1950, **43**, 308.
5. Dornbush, A. C., Personal communication.
6. McVay, L. V., Jr., Laird, R. L., and Sprunt, D. H., A preliminary report of the successful treatment of amebiasis with aureomycin. Science, 1949, **109**, 590.
7. Hewitt, R., Wallace, W., and White, E., The effect of aureomycin on *Endamoeba histolytica* in vitro. Science, 1950, **112**, 144.
8. Hall, W. H., The treatment of chronic human amebiasis with aureomycin. New England J. Med., 1951, **244**, 495.
9. Yeager, G. H., Byerly, W. L., Jr., and Holbrook, W. A., Jr., Streptomycin, aureomycin and chloromycetin: experimental and clinical comparison. Ann. Surg., 1949, **130**, 576.
10. Wright, L. T., Schreiber, H., Metzger, W. I., and Parker, J. W., An evaluation of aureomycin therapy in peritonitis. Surg., Gynec. & Obst., 1951, **92**, 661.

THE RATE OF REMOVAL OF RADIOACTIVE IRON FROM THE PLASMA—AN INDEX OF ERYTHROPOIESIS[1]

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Transport iron normally exists in the plasma as ferric beta-1-globulinate. Since in most individuals the greater part of the body iron is incorporated in the hemoglobin of the red blood cells, it is probable that changes in the production and destruction of these erythrocytes will be reflected in the turnover of plasma iron. Radioactive iron of high specific activity is particularly useful for a study of iron metabolism since it can be injected intravenously in tracer amounts without changing the steady state of the organism; the turnover of this radioiron will then mirror the metabolism of the iron in the body.

Flexner, Vosburgh, and Cowie (1) demonstrated that shortly after the intravenous injection of radioiron into guinea pigs, all the tagged iron in the plasma was in combination with beta-1-globulin. Following the injection of small quantities of such labelled plasma into other guinea pigs, the circulating plasma radioiron level decreased exponentially for the duration of the experiment. Labelled ferric beta-1-globulinate was also produced *in vitro* by the above investigators by incubating radioactive ferric chloride with a small amount of plasma. However, the intravenous injection of this mixture into guinea pigs was followed by a rapid loss of the plasma radioiron for about 30 minutes, following which the decrease again became exponential. The early rapid loss was presumably due to incomplete combination of the iron with the beta-1-globulin of the plasma and the diffusion of part of the injected iron into the tissues.

Similar results in humans utilizing *in vitro* tagged plasma were reported by Huff, Hennessy and Lawrence (2) and Huff and associates (3). Although in most instances, *in vitro* incubation of radioiron with patients' plasma resulted in a constant rate of disappearance of the radioactivity from the plasma following its injection intravenously, occasionally exponential disappearance rates were not obtained; the latter were thought to be due either to insufficient metal combining protein in the plasma or to the presence of an abnormal protein.

The following investigations of the plasma iron disappearance rate were undertaken to elucidate one phase of iron metabolism in anemic individuals in whom the anemia was due to bone marrow hypofunction; other patients with blood dyscrasias were also studied for comparison. A solution of plasma fraction IV–7 [2] was utilized for incubation with the radioactive iron since it was found that patients' plasma was on the whole unsatisfactory, due to variations in the latent iron binding capacities of such plasmas (4).

METHODS

Five microcuries of Fe^{59},[3] as ferric chloride, were prepared for injection by introducing about 0.05 ml. of the radioactive iron solution (specific activity 1.3 mc./mg.) into a rubber capped test tube, to which enough 4% sodium citrate was added to bring the pH between 6.5 and 7.0 (approximately 5 ml.). The mixture was autoclaved and 1 ml. of plasma fraction IV–7 containing 25 gm. of protein per 100 ml. was added with sterile precautions and incubated with occasional shaking at 37° C. for 30 minutes. During this period all of the iron combined with the protein, as proved by dialysis. Five ml. of this mixture were injected intravenously into suitable subjects in the post-absorptive state and the radioactivity of

[1] Aided by grants from the Anna Ruth Lowenberg and Albert A. List Funds.

[2] Obtained from the Department of Physical Chemistry, Harvard Medical School, through the courtesy of Dr. E. J. Cohn.

[3] The Fe^{59} initially used in this study was obtained through the courtesy of Dr. John H. Lawrence. The high specific activity Fe^{59} subsequently used in the cases of marrow aplasia was obtained on allocation from the Oak Ridge National Laboratories, through the Atomic Energy Commission.

successive samples of plasma measured. There were no untoward reactions to this procedure.

Samples of plasma were prepared by centrifugation of heparinized venous blood obtained at 10 to 30 minute intervals over a two to six hour period following the injection of the radioactive material. Total iron and latent binding capacity of the plasma (5, 6) were determined on at least two of the samples. Aliquots of 1 to 4 ml. were pipetted in duplicate into porcelain crucibles, to each of which were added 5 mg. of carrier iron as ferric chloride. Specimens were dried in an oven at 70° C. and ashed in a muffle furnace at 600° C. for 10 hours. The residue was dissolved in a few ml. of concentrated hydrochloride acid, evaporated almost to dryness and transferred quantitatively with distilled water into electroplating cells. A small amount of ascorbic acid was added to convert the iron to the ferrous form; the solution was then made slightly alkaline with concentrated ammonium hydroxide and buffered with 3 ml. of saturated sodium citrate. The iron was electroplated onto a thin copper disc 2.5 cm. in diameter, using a rotating platinum anode, at 1 ampere and 10 volts until the thiocyanate spot test for iron in the solution was negative (about four to five hours).[4] All the specimens were plated in duplicate.

The amount of radioactive iron electroplated on each disc was determined using an end window (mica, 1.5 mg./cm^2.) Geiger Müller tube; the background was about 1 count per second and the overall sensitivity was approximately 5,000 counts per second per microcurie Fe^{59}. The counts per disc ranged from 3 to 100 counts per second and were determined by observing the time required to accumulate 8,192 counts.

RESULTS

The disappearance of the injected labelled ferric beta-1-globulinate from the circulating plasma was exponential in all cases for the period of time followed. On semilogarithmic paper a straight line plot could be obtained and the rate of decrease in radioactivity determined by the slope of the line. For purposes of this report, this rate is expressed in terms of the half-time of disappearance ($T\frac{1}{2}$), *i.e.*, the time in minutes for the radioactivity of the plasma to be decreased to 50% of its initial value.

Values for absolute plasma iron turnover per unit time may be calculated from the radioiron disappearance data and the total plasma iron (3). Our results agree in general with those of Huff and his coworkers; several factors may influence this absolute iron turnover and form the basis of another report.

Studies on eight normal adult individuals and 32 patients with various blood dyscrasias comprise this report. From the data obtained in the former group, the normal pattern of plasma iron disappearance was derived. No attempt was made to ascertain the influence of various subsidiary factors as age, weight, sex, metabolic rate, and diet in essentially normal persons that could perhaps have some influence upon the plasma clearance data. These factors may require elucidation in the future. However, there is reason to believe that such variables will not have a decisive influence upon the results in the present study.

The results obtained are summarized in Table I and Figures 1, 2, and 3. The half-time of disappearance of the radioiron in the normals ranged from 70 to 140 minutes with an average of 90 minutes.

Three adult patients with chronic aplastic anemia (cases 9, 10, 11), in whom the diagnosis had been made on the basis of the usual clinical and laboratory data, were studied. Each had received 150 or more transfusions over a period of several years, and there was a uniform darkening of the skin and no splenomegaly. Skin biopsies revealed hemosiderosis. The serum iron was elevated and there was complete saturation of the beta-1-globulin. The disappearance of radioiron from the plasma was markedly prolonged with half-time values of 220, 260, and 270 minutes, respectively. A fourth patient (case 12) with a hypoplastic anemia and a superimposed hemolytic component who had received 15 transfusions totalling 7,500 ml. of blood, similarly showed a prolonged half-time of disappearance of 215 minutes. A few weeks following splenectomy, this value remained essentially unchanged at 225 minutes.

In addition, two patients, aged 10 and 22 years (cases 13 and 14) with a syndrome similar to aplastic anemia, namely, chronic aregenerative anemia, who had been transfused approximately bimonthly since birth were studied. They both showed a paucity of red cell precursors in the bone marrow, a severe normochromic anemia with normal white cells and platelets in the peripheral blood and the usual evidence of widespread hemosiderosis. The serum iron was elevated in both and the beta-1-globulin was completely saturated. The half-time of disappearance in both these cases was markedly prolonged to 205 and 245 minutes.

Another group of subjects showing bone mar-

[4] This method was suggested by Dr. Paul Hahn.

row aplasia or hypoplasia but associated with massive splenomegaly and myeloid metaplasia of the spleen was investigated. This series consisted of seven patients (cases 15 through 21) with myelofibrosis in whom the diagnosis was proved by bone marrow biopsy and splenic puncture. In six of these the rate of removal of radioiron from the plasma ranged from a half-time value of 20 minutes to 120 minutes; *i.e.*, 20, 25, 35, 95, 95, and 120 minutes. Two of this group (cases 20 and 21) received localized irradiation to the spleen (250r and 650r, respectively), following which there was a decrease in splenic size; concomitantly, the time of disappearance increased from 30 to 60 and from 20 to 210 minutes, respectively. The two patients with myelofibrosis (cases 18 and 15) who had received large numbers of blood transfusions, 125 and 110 units of blood, respectively, had skin biopsies which were positive for hemosiderin; the serum irons were elevated and the

TABLE I

Case no.	Diagnosis	Sex	Age (*yrs.*)	Hematocrit (%)	Transfusions (*blood-ml.* $\times 10^3$)	Serum iron (*gamma* %)	Plasma Fe^{59} disappearance ($T\frac{1}{2}$-*min.*)
1	Normal	M	44	48	0	50	100
2	Normal	M	46	40	0	90	70
3	Normal	M	49	50	0	110	80
4	Normal	M	49	42	0	95	70
5	Normal	M	38	41	0	130	140
6	Normal	M	32	43	0	110	100
7	Normal	M	50	46	0	80	95
8	Normal	M	52	40	0	100	75
9	Aplastic anemia	M	16	30	75	153*	220
10	Aplastic anemia	F	60	13	115	205*	260
11	Aplastic anemia	F	52	32	105	288*	270
12	Hypoplastic anemia	M	49	30	7.5	272*	215
12	(post splenectomy)	M	49	38	7.5	212*	225
13	Aregenerative anemia	M	10	31	160	268*	205
14	Aregenerative anemia	M	22	26	140	178*	245
15	Myelofibrosis	M	39	24	110	185*	213
16	Myelofibrosis	F	42	28	0	84	120
17	Myelofibrosis	F	56	21	1.5	137	95
18	Myelofibrosis	F	62	23	125	193*	95
19	Myelofibrosis	F	46	36	0	85	35
20	Myelofibrosis	M	72	32	0	112	25
20	(after splenic irradiation)	M	72	32	0	90	60
21	Myelofibrosis	M	53	31	2.5	125	20
21	(after splenic irradiation)	M	53	27	2.5	143	210
22	Polycythemia vera	M	30	52	0	105	60
23	Polycythemia vera	M	50	80	0	113	20
24	Polycythemia vera	M	37	63	0	55	11
25	Polycythemia vera	M	47	53	0	120	45
26	Polycythemia vera	F	51	51	0	120	30
26	(after 5 mc. P^{32})	F	51	48	0	130	145
27	Pernicious anemia	F	76	31	1	235*	40
27	(after vit. B_{12})	F	76	37	1	118	90
28	Pernicious anemia	F	74	26	0	265*	30
28	(after vit. B_{12})	F	74	38	0	78	55
29	Acquired hemolytic anemia	F	51	22	5	150	25
30	Sickle cell anemia	F	48	22	5.5	188	35
31	Chronic lymphatic leukemia	F	46	26	1.5	108	120
32	Reticulum cell leukemia	F	44	21	35	118	265
33	Chronic lymphatic leukemia	F	60	35	7.5	102	68
33	(after cortisone)	F	60	37	8.0	112	36
34	Hypochromic anemia	F	30	27	0	26	30
35	Hypochromic anemia	F	55	35	0	25	60
36	Myxedema	M	49	40	0	148	85
37	Uremia	F	59	24	0	195	185
38	Lymphosarcoma (post HN_2)	F	60	27	2	115	290
39	Disseminated lupus erythematosus	F	40	22	0	77	88
40	Lymphosarcoma	M	53	21	2.5	133	85

* Beta-1-globulin 100% saturated.

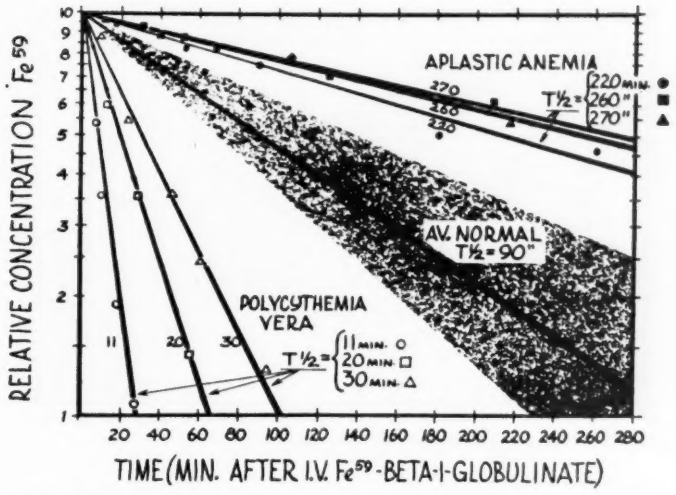

FIG. 1. COMPARISON OF THE PLASMA RADIOIRON DISAPPEARANCE IN THREE PATIENTS WITH APLASTIC ANEMIA (CASES 9, 10, 11), T½ = 220, 260, AND 270 MIN., WITH THREE PATIENTS WITH POLYCYTHEMIA VERA (CASES 24, 23, 26), T½ = 11, 20, AND 30 MIN.

Shaded area is normal range with average T½ = 90 min. Note the very rapid half-times of disappearance in the presence of hyperactive erythropoiesis, and the markedly prolonged T½ when red cell production is diminished or absent.

latent binding capacities of the sera were zero. In the former case, the half-time of disappearance was 95 minutes while in the latter it was markedly prolonged to 210 minutes. This patient was the only member of the myelofibrosis group to have an abnormally prolonged T½. Subsequent postmortem examination revealed marked myeloid metaplasia of the spleen and widespread generalized hemosiderosis.

In two cases of chronic lymphatic leukemia the half-times of disappearance were 120 and 68 minutes. Following one month of cortisone therapy the value in the latter subject (case 33) had decreased to 36 minutes. During this time there was a reticulocytosis of 9%, a slight increase in the packed cell volume and in normoblasts in the bone marrow. Another patient with reticulum cell leukemia who had been splenectomized and had received 70 transfusions, had a T½ increased to 265 minutes; a sternal marrow examination done at that time revealed only 3% of erythroid elements.

The clearance of the tagged iron from the plasma was increased in polycythemia vera. Five patients with the typical clinical and laboratory findings of idiopathic polycythemia had half-time values of 11, 20, 30, 45, and 60 minutes. One of these patients (case 26) was treated with 5 millicuries of radioactive phosphorus and one month after the injection, the T½ became increased from 30 to 145 minutes.

A patient with acquired hemolytic anemia (case 29) who had a severe anemia, splenomegaly, reticulocytosis, increased urinary and fecal urobilinogen, a positive Coombs' test and a markedly erythro-normoblastic marrow, had a T½ of 25 minutes. In another patient with the hemolytic syndrome of sickle cell anemia, the T½ was 35 minutes.

Two women with severe hypochromic microcytic anemia (cases 34 and 35) and low serum iron concentrations had rapid half-times of disappearance of 30 and 60 minutes.

Two patients over 70 years of age with pernicious anemia in relapse who had severe macrocytic anemia and combined system disease, showed increased half-time rates of 30 and 40 minutes. Six weeks following continuous therapy with vitamin

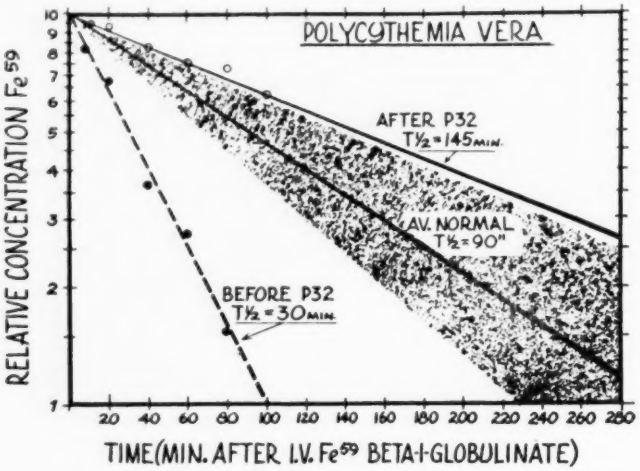

FIG. 2. PATIENT WITH POLYCYTHEMIA VERA (CASE 26) WHO RECEIVED 5 MILLICURIES P^{32} INTRAVENOUSLY

Note the increase in the T½ from a pretreatment value of 30 min. to 145 min. one month later. This illustrates the known effect of P^{32} in depressing erythropoiesis.

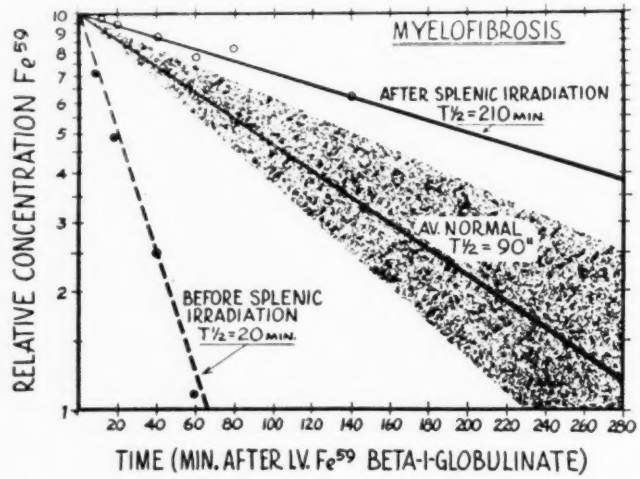

FIG. 3. COMPARISON OF THE HALF-TIME OF DISAPPEARANCE OF PLASMA RADIOIRON BEFORE AND AFTER SPLENIC IRRADIATION IN A PATIENT WITH MYELOFIBROSIS AND MARKED SPLENOMEGALY

From a pretreatment half-time value of 20 min. following 650r to the spleen, the disappearance rate became prolonged to 210 min. The importance of the spleen as a site for extra-medullary erythropoiesis in this syndrome is thus indicated and the influence of the degree of red cell production on the plasma radioiron clearance is demonstrated.

B_{12}, the anemia improved, the marrow became normal and the T½ became prolonged to 55 and 90 minutes respectively.

A miscellaneous group of patients was also examined. The half-time of disappearance in the various cases was as follows: severe myxedema, 85 minutes; uremia, 185 minutes; disseminated lupus erythematosus, 88 minutes; lymphosarcoma, 85 minutes. Another patient with lymphosarcoma who had received 2,000 ml. of blood, was given nitrogen mustard therapy; two weeks later the T½ was greatly prolonged to 290 minutes. A sternal marrow examination done before treatment was normally cellular with 20% erythroid elements. Following HN_2 therapy at the time of the studies with radioiron, the marrow was markedly hypoplastic with only 3% of red cell precursors.

DISCUSSION

In early experiments, the radioiron was incubated *in vitro* with the patient's own plasma prior to its intravenous injection. Although this technique gave reliable results in many individuals, it was observed that in subjects with hemosiderosis, the radioactive iron was lost from the plasma at a very rapid rate. This was found to be due to the fact that the beta-1-globulin of the plasma of these patients was already saturated with iron, hence combination between the radioiron and the plasma globulin did not occur. The iron injected thus remained in the ionic form and its rate of disappearance from the blood stream was markedly different from that of iron bound to globulin (4). The initial rapid loss of the tagged iron is probably due to diffusion into the intercellular space whereas the subsequent exponential disappearance of globulin bound iron results in part from the incorporation of the metal into hematopoietic foci, preparatory to hemoglobin synthesis, and deposition into the miscible iron pool.

To obviate this variation in combining capacity of different plasmas, a fairly pure source of beta-1-globulin was utilized to prepare the iron proteinate. The plasma fraction IV-7 (Cohn) as supplied is concentrated approximately 70-fold over normal plasma with respect to the metal combining globulin. When the radioiron is incubated with fraction IV-7 all radioiron disappearance rates are exponential irrespective of plasma iron concentration and the concentration and saturation of the circulating beta-1-globulin.

The nature of the radioiron disappearance curve deserves comment. Although no data for any period greater than six hours are available due to experimental limitations, it seems unlikely that any deviation from the exponential removal would occur. If this should happen, one would have to assume either an interruption of the steady state of the iron cycle or a feed-back of injected iron which has left the plasma. In the former instance, a new equilibrium would probably be reached and the function would again become exponential although possibly with a different rate constant. If feed-back is considered a possibility, this would imply a completion of the marrow or tissue store cycle which is highly improbable for the first few weeks, at least.

The rate of disappearance may be an average of several similar rates which are in turn dependent upon the uptake of iron by separate end organs, *i.e.*, the erythropoietic tissue, the fixed iron stores and the available iron pool; or perhaps in a particular metabolic state, one rate, exponential in nature, so dominates the plasma iron turnover that other rates become insignificant. Thus, in marrow aplasia where only a few percent of an injected dose of radioactive iron ultimately appears in the red cells, the disappearance rate is influenced largely by the uptake of iron by the tissues; iron leaves the plasma at a very slow rate in the blood dyscrasias in which the production of red cells is grossly impaired, as in aplastic and aregenerative anemia (7–9). On the other hand, in marrow hyperplasia, most of the radioiron becomes incorporated into the circulating erythrocytes (7–9); the rate of disappearance in this case is dependent in the main upon erythropoiesis, so that the presence of greater than normal numbers of red cell precursors, such as are known to exist in the bone marrow of polycythemia vera, hemolytic anemia and iron deficiency anemia, is accompanied by a rapid clearance of iron. These findings suggest that the turnover of the plasma iron as measured by the radioactive technique is an index of the degree of erythropoiesis in the body.

In pernicious anemia in relapse, in view of the megaloblastic arrest of the marrow a decreased rate of plasma iron disappearance might be antici-

pated. However, the values in two patients (cases 27 and 28) were quite rapid, 40 and 30 minutes, respectively. Six weeks following vitamin B_{12} therapy when the bone marrows were normal and the peripheral blood had returned nearly to normal, the study was repeated and respective rates of 90 and 55 minutes were obtained. In view of the high serum iron, complete saturation of the metal combining globulin and tissue hemosiderosis that exists in this disease, abnormal and rapid iron storage may play a role in the increased rate of plasma iron disappearance. Another possible explanation is the avidity of the marrow for iron in these cases because of actual increased erythropoiesis. The gross morphologic picture of red marrow replacing the normally yellow marrow areas coincides with this idea. It is possible that the erythrocytes and red cell precursors which are formed in pernicious anemia are broken down *in situ*. This is suggested by an excretion of urobilinogen far above that which would be derived from ordinary hemolysis in this disease. In this regard, London and West (10) have shown, using N^{15} labelled glycine in a case of pernicious anemia in relapse, that 40% of the urobilinogen appearing in the stool was not derived from circulating erythrocytes.

The bone marrow in myelofibrosis is hypocellular and a large portion of the marrow cavity is replaced by fibrous tissue or bone. There is usually a progressive splenomegaly with the spleen gradually assuming the erythropoietic function. Because of this compensatory metaplasia in the spleen, relatively good hemoglobin levels may be maintained for long periods of time. In view of the hypoplastic, fibrotic bone marrow, a slow rate of clearance of iron could be expected; however, most of the rates were rapid or within the normal range in the cases studied. This may be due to active red cell production in the spleen with, in some cases, a concomitant increased destruction of erythrocytes to account for the anemia. An elevation in the fecal and urinary urobilinogen was present in these latter cases and is suggestive confirmatory evidence of such a process. One of these patients (case 21) who had received a total of 650r to the spleen in a three week period, died subsequently. At autopsy the spleen showed very slight erythropoiesis contrary to that usually observed in myelofibrosis of advanced degree with myeloid metaplasia in the spleen. The prolongation of the half-time of disappearance of the plasma radioiron by splenic irradiation in the two patients mentioned above, demonstrates the effect of erythropoietic activity on plasma radioiron clearance. Furthermore, it emphasizes the importance of the spleen as a blood forming organ in this syndrome. The only patient of this group with a prolonged rate of disappearance was case 15, in whom there was a marked degree of exogenous hemochromatosis, secondary to multiple blood transfusions.

The influence of increased iron stores upon the rate of radioiron disappearance from the plasma is important. Data bearing on a closely allied problem have been reported by Finch and associates (7, 11). They found that in patients with endogenous hemochromatosis, radioiron utilization is profoundly depressed while erythrocyte production proceeds normally. Similarly, in normal subjects fed large quantities of iron for fairly long periods, the utilization of iron is reduced. Again, in iron loading experiments in dogs, comparison of pre- and post-loading utilization curves showed a markedly decreased uptake in the latter. It appears fairly definite therefore that increased iron stores depress the utilization of radioiron for hemoglobin production. Increased iron stores may similarly affect the rate of disappearance of radioactive iron from the plasma. Our data are as yet too meager to resolve this question. As has been noted above, case 15 with myelofibrosis and hemosiderosis exhibited a markedly prolonged $T\frac{1}{2}$; however, another subject (case 18) with the same combined syndrome, who had received an even larger number of blood transfusions, had a normal iron disappearance rate. All the patients with aregenerative and aplastic anemia had hemosiderosis, but it appears more likely that the marrow aplasia rather than the enlarged iron depots is the primary factor in prolonging the disappearance rate in these cases.

Although it is possible that iron stores may affect the radioiron disappearance rate, it has been demonstrated that marked variations in plasma iron clearance may occur in the same patient with no change in iron stores. Thus, the patient with polycythemia vera (case 26) who received 5 millicuries of P^{32} exhibited a pretreatment of $T\frac{1}{2}$ of 30 minutes which then became prolonged to 145 minutes one month later. At the same time, the

packed cell volume decreased from 51% to 48% and the per cent of erythroid elements in the sternal marrow similarly showed a reduction from 60% to 25%. The decrease in the half-time of disappearance was then a gauge of the effectiveness of bone marrow irradiation in depressing erythropoiesis. In view of the very slight decrease in hematocrit and no essential change in serum iron concentration (see Table I), it is clear that no alteration in iron stores was likely.

As has been previously noted, two patients with myelofibrosis had received localized irradiation to the spleen immediately following which the radioiron disappearance rates decreased. No bleeding was observed nor were any transfusions administered in the interim. During this period, the packed cell volumes decreased moderately while the serum iron remained essentially unchanged. Here again in the presence of constant iron stores a marked decrease occurred in the turnover of radioactive iron from the plasma.

It is apparent that the removal of radioiron from the plasma is an index of red cell production in the body in patients with normal iron stores. A change in the steady state of the turnover of iron will produce an effect on the iron clearance from the plasma. Following the establishment of a new equilibrium, a new iron disappearance rate constant will be obtained. The rate of iron disappearance may be of value as a diagnostic test, particularly when combined with a study of the utilization of iron by the red cells. However, where large numbers of transfusions have been given, the rate of disappearance may be influenced by the increased fixed iron stores. The latter problem is currently being investigated in this laboratory.

SUMMARY

1. A method for the determination of the rate of disappearance of iron from the plasma using radioactive iron and a solution of beta-1-globulin has been described.

2. A correlation between erythropoiesis and the rate of disappearance of radioiron from the plasma has been established.

3. In aplasia of the bone marrow, the rate of removal of radioiron from the plasma is markedly prolonged. Increased marrow erythropoiesis as occurs in polycythemia vera, hemolytic anemia and severe hypochromic anemia results in an increased rate of removal of radioiron from the plasma.

4. Greater than normal iron stores, as occurs in hemosiderosis, may prolong the plasma radioiron turnover.

5. It is suggested that with normal body iron stores the half-time of plasma radioiron disappearance is a sensitive measure of the integrity of the erythropoietic tissue of the body.

REFERENCES

1. Flexner, L. B., Vosburgh, G. J., and Cowie, D. B., Capillary permeability; rate of transcapillary exchange of iron added to plasma as radioactive ferric beta-1-globulinate. Am. J. Physiol., 1948, **153**, 503.
2. Huff, R., Hennessy, T., and Lawrence, J. H., Iron metabolism studies in normal subjects and in patients having blood dyscrasias. J. Clin. Invest., 1949, **28**, 790.
3. Huff, R. L., Hennessy, T. G., Austin, R. E., Garcia, J. F., Roberts, B. M., and Lawrence, J. H., Plasma and red cell iron turnover in normal subjects and in patients having various hematopoietic disorders. J. Clin. Invest., 1950, **29**, 1041.
4. Wasserman, L. R., and Rashkoff, I. A., Unpublished data.
5. Rath, C. E., and Finch, C. A., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXXVIII. Serum iron transport. Measurement of iron binding capacity of serum in men. J. Clin. Invest., 1949, **28**, 79.
6. Cartwright, G. E., and Wintrobe, M. M., Chemical, clinical and immunological studies on the products of human plasma fractionation. XXXIX. The anemia of infection. Studies on the iron binding capacity of serum. J. Clin. Invest., 1949, **28**, 86.
7. Finch, C. A., Gibson, J. G., II, Peacock, W. C., and Fluharty, R. G., Iron metabolism. Utilization of intravenous radioactive iron. Blood, 1949, **4**, 905.
8. Dubach, R., Collender, S. T. E., and Moore, C. V., Studies in iron transportation and metabolism. VI. Absorption of radioactive iron in patients with fever and with anemias of varied etiology. Blood, 1948, **3**, 526.
9. Rashkoff, I. A., Wasserman, L. R., Leavitt, D., and Mayer, J., Radioactive iron studies in anemia. Bull. N. Y. Acad. Med., 1951, **27**, 387.
10. London, I. M., and West, R., The formation of bile pigment in pernicious anemia. J. Biol. Chem., 1950, **184**, 359.
11. Finch, C. A., Hegstead, M., Kinney, T. D., Thomas, E. D., Rath, C. E., Haskins, D., Finch, S. and Fluharty, R. G., Iron metabolism. The pathophysiology of iron storage. Blood, 1950, **5**, 983.

LUNG FUNCTION STUDIES. VIII. ANALYSIS OF ALVEOLAR VENTILATION BY PULMONARY N_2 CLEARANCE CURVES [1,2]

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A comprehensive analysis of pulmonary function must include measurements of pulmonary ventilation, diffusion, and circulation. An accurate measurement of alveolar ventilation, independent of diffusion and circulation, is desirable. To date, no method has been wholly satisfactory (3). One approach has been by study of the time course of equilibration of alveolar gas with a "foreign" inspired gas, such as H_2 (4), He (5–7) or pure O_2 (8–15). A principal difficulty has been to differentiate between the effects of an enlarged respiratory dead space and of uneven alveolar ventilation, both of which can retard the completion of equilibration.

The development by Lilly and Hervey of the N_2 meter, for continuous analysis of N_2 concentration of respired gas, permits an automatic breath-by-breath analysis of gas expired during and after the change from breathing air to uninterrupted breathing of O_2. The ensuring progressive elimination of pulmonary N_2 represents the process of equilibration with a N_2-free gas, and may be analyzed in terms of alveolar ventilation. This paper presents the pulmonary N_2 clearance curves obtained from healthy persons and patients with cardiorespiratory disease, and a method for analysis of these curves which yields a comprehensive picture of the volume and time characteristics of alveolar ventilation. The lungs of healthy subjects, to a small degree, and of persons with cardiorespiratory disease to a greater degree, are ventilated unevenly; *i.e.*, certain regions appear to be ventilated more rapidly than other regions. The respective volumes and ventilation rates of these several regions can be determined, as well as the volume and average ventilation rate of the lungs as a whole. The extent of uneven ventilation can be measured quantitatively; this measurement is uninfluenced by the magnitude of the respiratory dead space.

Although this method is laborious at present, it should be useful for investigation of pulmonary physiology, and has served as a standard for simpler methods of studying the uniformity of alveolar ventilation.

THEORETICAL CONSIDERATIONS

Most simply, the lungs could be represented by a bellows which is uniformly ventilated, *i.e.*, inspired gas is distributed evenly to and mixed instantly with all the gas previously present in the bellows. For simplicity, it will be assumed for the moment that inspired gas contains no N_2, and that there is no transfer of N_2 from blood and tissue. The basic equation presented by Darling, Cournand and Richards (9) to describe the progressive reduction of alveolar N_2 concentration during the breathing of a N_2-free gas (O_2) in such a system is:[4]

$$F_{A_n} = F_{A_0} w^n, \qquad (1)$$

where

F = fractional gas concentration. Throughout this paper it refers to N_2.

F_{A_0} = alveolar N_2 concentration before O_2 inhalation. This is assumed to be 80% during breathing of air.

F_{A_n} = alveolar N_2 concentration after n breaths of pure O_2.

$w = \dfrac{V_L}{V_L + (V_T - V_D)}$. This will be called the alveolar dilution factor.

V_L = volume in which the N_2 is contained at the end of expiration = functional residual capacity = volume of alveolar spaces + volume of respiratory dead space.

V_T = tidal volume.

V_D = volume of respiratory dead space.

Equation (*1*) requires values for alveolar N_2 concentration. These were obtained by Darling from the terminal part of forced-expiration alveolar gas samples. However, recent studies have shown that the N_2 concentration of alveolar

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[4] The symbols used are those recently proposed to achieve standardization of respiratory terminology (16).

gas expired after O_2 inhalation varies even during one exhalation, especially in subjects with pulmonary disease (17, 18). Thus one is faced with a variety of different alveolar N_2 concentrations, none of which is necessarily correct for use in equation (*1*). However, the mean N_2 concentration of any single expiration has only one value and is therefore preferable for measurement. It is related to the alveolar concentration as follows:

$$\bar{F}_E V_T = \bar{F}_A (V_T - V_D) + F_D V_D, \qquad (2)$$

where

$\bar{F}_E$ = mean expired N_2 concentration.

F_D = N_2 concentration in the dead space gas at end-inspiration.

$\bar{F}_A$ = mean N_2 concentration of expired alveolar gas. In a uniformly ventilated system, this equals end-inspiratory alveolar concentration, F_A in equation (*1*). Because F_D during O_2 inhalation is practically zero, $F_D V_D$ equals zero.

Rearranging,

$$\bar{F}_E = \bar{F}_A \frac{V_T - V_D}{V_T}. \qquad (3)$$

Let $\frac{V_T - V_D}{V_T}$ equal r or the dead space dilution factor.

From equation (*1*), one can substitute for $\bar{F}_A$ in equation (*3*).

$$\bar{F}_{E_n} = F_{A_0} w^n r \text{ or } \log \bar{F}_{E_n} = \log r F_{A_0} + n \log w. \qquad (4)$$

Thus $\log \bar{F}_{E_n}$ plotted against n yields a straight line, the value of which at n = 0 equals $\log r F_{A_0}$, and the slope of which equals log w. Knowing F_{A_0}, $\bar{F}_{E_0}$, and V_T, one obtains $(V_T - V_D)$ from equation (*3*); from the value and definition of w, and the value of $(V_T - V_D)$, the value of V_L is obtained. Thus all the ventilatory factors can be determined from measurements which are relatively easily and accurately made, *viz.*, F_{A_0}, V_T, n and $\bar{F}_{E_n}$. However, equation (*4*), describing the ventilation of a single bellows, was found to be inadequate for experimental use in almost all cases, principally because the lungs, even in normal subjects, were not evenly ventilated. Its derivation above is presented to clarify further equations for combinations of *several single* bellows, ventilated synchronously, which were found to fit the experimental data satisfactorily.

Since uneven ventilation is probably largely of a regional nature (19), equations were developed in terms of several independent lung regions or phases, within each of which ventilation is uniform (though not necessarily equal to that of other regions), and from which the separate expired tidal volumes are combined. Such equations will fit the experimental data.

For a two-phase system, the expired N_2 is represented by:

$$\bar{F}_E = \frac{V_{T_1} \bar{F}_{E_1} + V_{T_2} \bar{F}_{E_2}}{V_T} \qquad (5)$$

in which the arabic subscripts 1, 2, etc. denote different phases; when the number of respirations is also designated, it is given by either n or o, placed as a sub-subscript; and

V_{T_1}, V_{T_2} = tidal volumes of respective phases or regions.

$\bar{F}_{E_1}$, $\bar{F}_{E_2}$ = mean expired N_2 concentrations of V_{T_1} and V_{T_2}, respectively.

From equation (*4*), $\bar{F}_{E_{1_n}} = F_{A_{1_0}} w_1^n \frac{V_{T_1} - V_{D_1}}{V_{T_1}}$, in which $w_1 = \frac{V_{L_1}}{V_{L_1} + (V_{T_1} - V_{D_1})}$ and similarly for F_{E_2}, then

$$\bar{F}_{E_n} = F_{A_{1_0}} w_1^n \frac{V_{T_1} - V_{D_1}}{V_T} + F_{A_{2_0}} w_2^n \frac{V_{T_2} - V_{D_2}}{V_T}. \qquad (6)$$

Let $\frac{V_{T_1} - V_{D_1}}{V_T} = r_1$ and $\frac{V_{T_2} - V_{D_2}}{V_T} = r_2$, then

$$\bar{F}_{E_n} = F_{A_{1_0}} w_1^n r_1 + F_{A_{2_0}} w_2^n r_2 \qquad (7)$$

or

$$\log \bar{F}_{E_n} = \log (F_{A_{1_0}} w_1^n r_1 + F_{A_{2_0}} w_2^n r_2). \qquad (8)$$

For a system of three regions or phases

$$\log \bar{F}_{E_n} = \log (F_{A_{1_0}} w_1^n r_1 + F_{A_{2_0}} w_2^n r_2 + F_{A_{3_0}} w_3^n r_3). \qquad (9)$$

It will be remembered that w is a fractional value; hence $F_{A_0} w^n r$ approaches zero as n increases. If $w_3 < w_2 < w_1$, $\log \bar{F}_{E_n}$ will, as n increases, successively reflect the three components, then only the latter two, and finally only $F_{A_{1_0}} w_1^n r_1$, after which time $\log \bar{F}_{E_n}$ plotted against n is linear.

To this point, it was assumed that inspired gas was 100% O_2 and that there was no transfer of N_2 from blood and tissue. When the concentration of N_2 in inspired gas is F_I, equation (*7*), for example, becomes

$$\bar{F}_{E_n} = (F_{A_{1_0}} - F_I) w_1^n r_1 + (F_{A_{2_0}} - F_I) w_2^n r_2 + F_I. \qquad (10)$$

As the N_2 tension of alveolar gas is lowered, N_2 from blood and tissues enters the alveoli and is eliminated in the expired gas. The exact modification of equation (*10*) which is required to include this N_2 is not known. However, the rate of elimination of blood and tissue N_2 must start at zero, increase and later decrease. It is probable that a maximum occurs during the later part of pulmonary N_2 elimination, several minutes after the start of O_2 inhalation (20). However, the quantities of blood and tissue N_2 eliminated are small, being about 200–300 ml. in a seven minute period of O_2 inhalation (21). We have assumed for purposes of calculation that this is eliminated at a constant rate so that equation (*10*) becomes

$$\bar{F}_{E_n} = (F_{A_{1_0}} - F_I) w_1^n r_1 + (F_{A_{2_0}} - F_I) w_2^n r_2 + F_I + F_b, \qquad (11)$$

where Fb represents the correction for blood and tissue N_2 appearing in the expired gas. The total amount of N_2 eliminated in seven minutes is estimated by the formula of Cournand (21), and Fb is calculated as follows:

$$F_b = \frac{\text{Total } N_2 \text{ eliminated in 7 min.}}{\text{No. of breaths in 7 min.} \times V_T}$$

Errors in this correction are of negligible importance early in the procedure. Later, as alveolar N_2 concentration becomes low, it may be incorrect and of some importance, as noted later.

Experimentally, the mean expired N_2 concentration of various expirations is measured. From these values the combined correction for inspired N_2 and N_2 eliminated from the blood, about 0.5–0.9%, is subtracted. The corrected values are then plotted on semi-logarithmic paper, the log of $\bar{F}_{E_n}$ being plotted against n. Such a curve is given in Figure 1, and the analysis into the exponential components by graphic methods is illustrated. The graphic

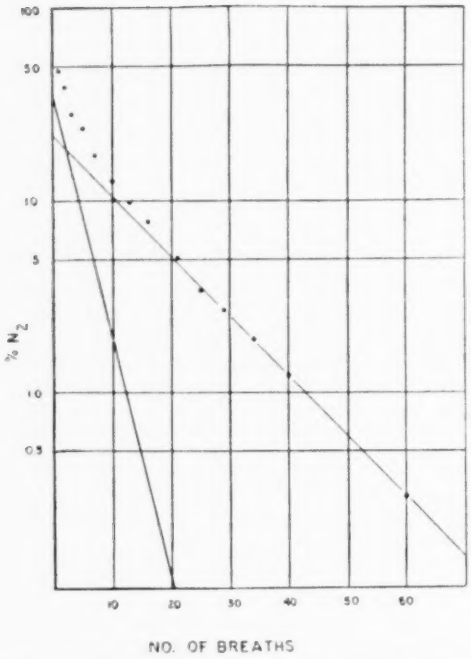

FIG. 1. GRAPHIC ANALYSIS OF DATA FROM HEALTHY ELDERLY SUBJECT T. H., ILLUSTRATING A TWO-PHASE CURVE.

The dots are mean expired N_2 concentration minus correction (0.6%) for inspired and blood N_2. A curve through all dots represents the sum of the two components. One component is determined by the less steep straight line, or $(F_{A_1} - F_I)w_1^n r_1$; the difference between this line and the dots is represented by the steeper straight line, $(F_{A_2} - F_I)w_2^n r_2$. See text.

analysis of the clearance curve is somewhat subjective. However, satisfactory precision is indicated by the reproducibility of measurements, and the magnitude of the derived dead space values, noted below. With large values of w, it is both necessary and possible to determine its magnitude to three figures. With small values of w, both the precision of determination and the effect of errors therein decrease. The analysis of the data by means of the preceding equations yields values of V_L, w, and $(V_T - V_D)$ for the respective components.

It is also important to know the ventilation rate of the lungs as a whole, the magnitude of uneven ventilation, and to make a ready comparison of these between individuals. This information can be obtained as follows from the data for the several components. Robertson and associates pointed out the relationship between the turnover rate and the average time a molecule remains in a continuously ventilated system (12). This approach can be applied to a cyclically ventilated system such as the lungs. The basic value is that of w. However, on first acquaintance, some difficulty may be experienced in its interpretation. There is a derived number by which the ventilation can be characterized, and which is perhaps more easily comprehended. This is the average number of breaths the N_2 molecules remain in a system, from which the N_2 is being completely washed out.[5] This, as defined in the footnote, can be shown to equal $\frac{1}{1-w}$, when $w = \frac{V_L}{V_L + (V_T - V_D)}$. The average number of breaths N_2 molecules remain is small in a "deeply" ventilated system (w is small), and large if "shallowly" ventilated (w approaches unity).

The average number of breaths N_2 molecules remain in a system having several components, each with different values of w (w_1, w_2, w_3) and in which the decimal fractions of the total lung volume (V_L) occupied by the partial lung volumes (V_{L_1}, V_{L_2}, V_{L_3}) are, respectively, f_1, f_2, f_3, equals $\frac{f_1}{1-w_1} + \frac{f_2}{1-w_2} + \frac{f_3}{1-w_3}$; this value hereafter is called the *actual* average breath number.

The magnitude of uneven ventilation can be derived as follows. For a system having certain values for V_L and $(V_T - V_D)$, the average number of breaths N_2 molecules remain can be shown to be minimal when the whole system is ventilated evenly.

If the effective tidal volume $(V_T - V_D)$ is *unevenly* distributed to various subdivisions of the total lung volume, the average number of breaths the N_2 molecules remain is increased. However, one can determine what this number would be if ventilation *were uniform, i.e.*, the *ideal* average breath number, and thereby acquire a standard for comparison with the *actual* average breath number.

It can be shown that $\frac{f_1}{w_1} + \frac{f_2}{w_2} + \frac{f_3}{w_3} = \frac{1}{W}$ in which f_1 and w_1, *etc.*, are as above and W = the value of the alveolar dilution factor which would obtain in a uniformly ventilated system having the same total V_L and $(V_T - V_D)$ as the components. The average number of breaths the N_2 molecules would remain in this uniformly ventilated system or the *ideal* average breath number is $\frac{1}{1-W}$

If uneven ventilation exists, its extent is given by the delay in N_2 elimination produced thereby, expressed as a percentage of the ideal situation.

Pulmonary N_2 clearance delay (%)

$$= \frac{\textit{Actual} \text{ average breath no.} - \textit{Ideal} \text{ average breath no.}}{\textit{Ideal} \text{ average breath no.}} \times 100.$$

The *time* intervals, in minutes, which correspond to the *actual* and *ideal* average breath numbers equal, respectively,

$$\frac{\text{Actual average breath no.}}{\text{Resp. frequency}} \text{ and } \frac{\text{Ideal average breath no.}}{\text{Resp. frequency}}$$

[5] The average number of breaths the N_2 molecules remain $= \frac{\sum_{i=1}^{n} i \times M_i}{n}$, where i = number of breaths; M = number of molecules remaining for i breaths; n = total number of molecules.

PROCEDURE

Details of apparatus have been presented previously (22, 23). In brief, the method consists of continuous analysis and photographic recording of (*1*) expiratory volume flow and (*2*) N_2 concentration of respired gases during and after the change from breathing room air to breathing oxygen, the N_2 content of which, 0.25% to 0.4%, was measured for each cylinder with the Van Slyke manometric apparatus. The accuracy of our N_2 meter as used here with reference values (O_2) recorded on each inspiration, is about $\pm 2\%$ N_2 at high concentrations, $\pm 1\%$ N_2 from about 30% to 10% N_2, and $\pm 0.5\%$ N_2 at lower concentrations. Inspired O_2 was delivered from a demand regulator, expired gas was passed through a flow meter, 30 inches of flexible rubber tubing (2 cm. I.D.) and the expiratory valve to a 120 L. compensating recording Tissot spirometer. The tubing-spirometer system was flushed previously with O_2; the gas expired into the spirometer during a seven minute period of breathing O_2 permitted measurement of functional residual capacity (FRC) by the method of Darling (24). Tidal volume and respiratory frequency were recorded spirometrically.

The subjects, after semireclining in bed with the trunk and head elevated 50–60° for at least 10 minutes, breathed O_2 quietly for seven minutes. Continuous recordings of N_2 concentration and volume flow of expired gas were made during the first two minutes of O_2 inhalation, and for one or two breaths at the end of each succeeding minute for seven minutes. The mean expired N_2 concentration of each breath was determined by replotting the simultaneous N_2 and flow curves as % N_2 versus volume instead of each versus time, as recorded. The area under this curve is quantity of N_2 expired, and the mean height equals mean N_2 concentration. This was determined graphically for 10–20 separate expirations.[6] Since accuracy of this procedure becomes less as N_2 concentrations decrease, a different procedure was used to measure later breaths in which alveolar (end-tidal) N_2 concentration was less than 5% N_2. Mean expired N_2 concentration was calculated as end-tidal concentration times the average value of $\frac{\text{mean \% } N_2}{\text{end-tidal \% } N_2}$ as determined by graphic integration of several prior breaths of similar volume. This calculation is strictly correct only when inspired gas contains no N_2; however, as used here, the error is negligible. Measurements were continued until the mean expired N_2 concentration was about 1% N_2. The tidal volumes during the period of N_2 measurements were corrected to BTPS and average values were used in calculation.

RESULTS

Healthy adults

Table I presents the results on 10 healthy young adults (seven male and three female) and on seven elderly male hospital patients, in whom the history, physical and radiological examinations gave no evidence of cardiorespiratory disease. The majority of the clearance curves could be described by an equation for two phases, each having different volumes and ventilation rates. The value of w, the alveolar dilution factor, is a measure of the effective ventilation per breath; as w becomes smaller, the effective ventilation increases, *i.e.*, greater dilution of alveolar gas occurs. The alveolar dilution factor of the major part, average about 80%, of the lung volume appeared to be of a moderate magnitude, having values of about 0.9. This is of the order to be expected from customary values of functional residual capacity, tidal and dead space volumes. However, a smaller part, about 20% of the lung volume, was relatively hyperventilated, having w values between about 0.5 and 0.85.

For the younger subjects, the average number of breaths N_2 molecules remained in the lungs (actual average breath number) varied from 5.9 to 9.7; the mean was 7.63 breaths, or 0.60 minutes. If alveolar ventilation had been uniform, N_2 molecules would have remained 6.38 breaths, or 0.50 minutes. Uneven ventilation was of moderate extent, producing an average pulmonary N_2 clearance delay of 21.1%, S.D. $\pm$ 11.9%.

The older subjects differed from the younger in several respects. The effective ventilation per breath of the major part of the lung volume tended to be less. The mean of the actual number of breaths N_2 molecules remained in the whole lung, 11.14 breaths, was significantly greater ($p < 0.01$) than for the younger subjects. This was a result of two factors. First, the average extent of uneven ventilation, shown by an average pulmonary N_2 clearance delay of 45.9%, was significantly larger ($p < 0.05$). Second, N_2 molecules would have remained a significantly greater ($p < 0.05$) number of breaths even if ventilation had been uniform, as shown by the average ideal breath number of 7.77. This is because the enlargement of functional residual capacity (V_L) was not accompanied by a corresponding increase in effective tidal volume ($V_T - V_D$). However, the average *time* N_2 molecules remained in the lungs, about 40 seconds, was not significantly prolonged in

[6] The graphic integration procedure requires several hours for each subject's record. Attempts, as yet not completely successful, have been made to devise an automatic integration apparatus which would reduce the measurement time to several minutes.

the older subjects because of their greater respiratory frequency.

Subjects with cardiorespiratory abnormality

Table II presents the results on 19 patients. These subjects were selected to present a wide variety of pulmonary disorders, both in nature and severity. Of four subjects with clinically varying severity of asthma, three had abnormally uneven ventilation, shown by pulmonary N_2 clearance delay values of 52, 95, and 190%. In the two most severe cases, the average number of breaths N_2 molecules actually remained was more than three times the normal values. One patient (V. J.) with clinically mild asthma (no symptoms or physical signs at the time of the examination) exhibited an abnormally small effective ventilation per breath, as shown by the large actual and ideal breath numbers, although the uniformity of ventilation was not abnormal (13% pulmonary N_2 clearance delay).

All five subjects with chronic pulmonary emphysema showed findings similar to each other. The effective ventilation per breath of the major part of the alveolar gas was very small, with w values exceeding 0.98. Even if ventilation had been uniform, the effective ventilation per breath would have been small, as shown by the abnormally great ideal breath numbers. This is the result of enlargement of the functional residual capacity. Pulmonary N_2 clearance delay was marked, ranging from 115 to 248%. These combined to produce a very slow average ventilation, the average time N_2 molecules actually remained amounting to several minutes.

The group of subjects with congestive heart failure, all with mitral valvular disease, exhibited varied patterns of ventilation. In three of the five, ventilation was abnormally uneven, with clearance delay values from 60 to 102%. The other two cases, despite similar normal values for N_2 clearance delay, showed certain differences. One case (J. P.) with a clinically minimal

TABLE I

Respiratory and pulmonary N_2 clearance data for young and old healthy subjects
(See text for explanation of headings)

Subj.	Sex	Age (yrs.)	Tidal vol. (ml.)	Resp. (per min.)	Ventilatory components						Average interval N_2 molecules remained				Pul. N_2 clearance delay (%)	FRC (ml.)	
					Phase 1		Phase 2		Phase 3		No. of breaths		Minutes				
					f_1	w_1	f_2	w_2	f_3	w_3	Actual	Ideal	Actual	Ideal		Darling	ΣV_L
H. B.	M	30	740	15	.94	.865	.06	.588	—	—	7.08	6.25	.47	.42	13	—	2,910
R. W.	M	26	700	10	.66	.864	.34	.680	—	—	5.92	4.76	.59	.48	24	—	1,930
S. K.	M	34	620	12	.74	.879	.26	.657	—	—	6.87	5.20	.57	.43	32	—	1,910
B. K.	M	30	557	15	.84	.886	.16	.758	—	—	7.98	7.25	.53	.48	10	2,310	2,370
H. K.	M	27	467	14	.88	.853	.12	.500	—	—	6.24	4.73	.45	.34	32	1,520	1,270
L. H.	M	30	558	18	.68	.896	.32	.722	—	—	7.66	5.86	.43	.33	31	2,240	2,380
C. W.	M	35	514	10	.85	.903	.13	.865	.03	.524	9.69	8.40	.97	.84	15	2,500	2,670
M. N.	F	28	453	13	1.00	.869	—	—	—	—	7.63	7.63	.59	.59	0	—	2,210
I. M.	F	22	510	12	.96	.893	.04	.537	—	—	9.00	7.70	.75	.64	17	2,760	2,560
D. R.	F	22	481	13	.84	.893	.16	.621	—	—	8.27	6.06	.64	.47	37	1,800	1,790
Mean		25.4	560	13.2	.84	.880	—	—	—	—	7.63	6.38	.60	.50	21.1	2,188	2,200
S.D.±											1.18	1.30	.16	.15	11.9		
T. H.	M	53	466	20	.74	.931	.26	.751	—	—	11.80	8.15	.59	.45	45	1,930	2,400
F. M.	M	56	330	21	1.00	.888	—	—	—	—	8.92	8.92	.43	.43	0	1,570	1,360
C. W.	M	61	550	11	.82	.899	.18	.613	—	—	8.49	5.82	.77	.53	46	3,510	3,270
G. R.	M	65	643	14	.72	.924	.23	.838	.05	.375	11.03	6.50	.80	.46	70	3,130	3,010
M. G.	M	65	464	15	.42	.929	.58	.864	—	—	10.17	9.10	.68	.61	12	1,940	2,350
J. F.	M	61	577	21	.69	.951	.26	.819	.05	.486	15.50	7.70	.74	.37	101	4,090	2,640
H. S.	M	81	620	16	.93	.922	.07	.554	—	—	12.06	8.20	.76	.51	47	3,250	3,390
Mean		64	521	16.9	.76	.921					11.14*	7.77*	.68	.48	45.9*	2,774	2,631
S.D.±											2.35	1.21	.13	.08	33.9		

* Mean value differs significantly by small sample method from mean value for young subjects.

TABLE II

Respiratory and pulmonary N_2 clearance data for subjects with cardiorespiratory disease

Subj.	Diagnosis	Sex	Age (yrs.)	Tidal vol. (ml.)	Resp. (per min.)	Ventilatory components						Average interval N_2 molecules remained				Pul. N_2 clearance delay (%)	FRC (ml.)	
						Phase 1		Phase 2		Phase 3		No. breaths		Minutes				
						f_1	w_1	f_2	w_2	f_3	w_3	Actual	Ideal	Actual	Ideal		Darling	ΣV_L
	Asthma																	
E. R.	mild	F	34	710	17	.46	.911	.54	.694	—	—	6.87	4.51	.41	.27	52	2,530	1,910
V. J.	mild	F	33	364	25	.71	.936	.29	.870	—	—	13.3	11.8	.53	.47	13	2,060	2,310
L. S.	moderate	M	16	525	22	.54	.979	.38	.950	.08	.722	33.5	17.2	1.5	.78	95	3,970	4,720
J. K.	severe	M	41	482	20	.53	.987	.47	.882	—	—	44.5	15.4	2.2	.77	190	3,250	3,750
	Emphysema																	
G. D.	moderate	M	70	437	17	.79	.980	.21	.844	—	—	40.7	18.9	2.4	1.1	115	4,600	4,370
N. R.	severe	M	58	312	22	.87	.990	.13	.820	—	—	87.5	27.8	4.0	1.3	215	4,680	3,710
J. C.	severe	M	59	395	20	.72	.991	.25	.926	.03	.622	83.4	24.4	4.2	1.2	240	4,670	4,600
W. A.	severe—asthma	M	60	497	16	.72	.981	.20	.862	.08	.603	39.7	11.4	2.6	0.74	248	3,880	3,440
S. F.	severe—asthma	M	66	467	19	.82	.981	.16	.858	.02	.418	44.5	16.7	2.3	0.88	166	4,250	4,180
	Congestive heart failure																	
J. P.	minimal	M	40	307	26	.86	.934	.14	.779	—	—	13.7	10.9	.53	.42	25	2,070	1,730
J. B.	moderate	M	39	574	13	.48	.953	.52	.771	—	—	12.5	6.67	.96	.52	89	2,400	2,170
G. B.	severe	F	39	451	30	.61	.847	.39	.626	—	—	5.0	3.90	.17	.13	29	1,580	1,030
E. W.	severe	F	36	320	32	.45	.972	.48	.926	.07	.752	23.4	14.7	.73	.46	60	2,330	2,700
G. W.	severe—with (?)silicosis	M	56	492	27	.78	.976	.22	.841	—	—	33.7	16.7	1.25	.62	102	4,570	5,560
B. T.	Bronchiectasis, L. lower lobectomy, 8 mos. pregnant	F	22	670	14	.17	.966	.75	.810	.08	.304	9.05	3.73	.65	.27	143	1,660	1,390
E. T.	Sarcoid-pulmonary cyst	F	26	400	31	.69	.973	.31	.850	—	—	27.6	14.3	.90	.46	93	3,330	2,640
T. C.	Pulmonary fibrosis	M	34	392	21	.48	.980	.47	.938	.05	.393	31.6	9.51	1.51	.45	233	2,620	2,760
L. W.	3 mos. post-pneumonectomy	M	65	430	23	.48	.961	.37	.892	.15	.635	16.1	7.75	.70	.34	108	1,850	1,750
A. G.	2 wks. post-pneumonectomy	M	54	556	24	.55	.952	.37	.885	.08	.498	14.5	7.40	.61	.31	97	2,700	2,040

degree of failure had an abnormally small effective ventilation per breath (large ideal and actual breath numbers), resulting primarily from a small tidal volume, 307 ml. The other (G. B.), with clinically severe pulmonary and peripheral congestion, had an abnormally great effective ventilation per breath. With a marked polypnea, this produced the most rapid pulmonary N_2 clearance observed.

The two cases examined after pneumonectomy for cancer were selected to see if uneven ventilation could occur in a single lung which was considered pre-operatively to be clinically normal. In both cases, uneven ventilation was marked, the clearance delay values, 108 and 97%, exceeding considerably the average of normal elderly men, 46%. However, these cases cannot be considered as normal single lungs. In both the remaining lung was somewhat overdistended, as indicated by tracheal displacement observed fluoroscopically and by residual volume /total lung capacity ratios of about 55%. Also, in case L. W. emphysematous changes were found at autopsy 13 months later. However, these show that a single lung can be ventilated unevenly.

Abnormally uneven ventilation was also found in three patients with other varied types of pulmonary disease. Subject B. T. further illustrates the fact that markedly uneven ventilation is not necessarily associated with a long absolute pulmonary clearance time. Uneven ventilation creates a delay beyond the value which would obtain with even ventilation. If the latter is small, because effective minute volume ($[V_T - V_D] \times$ resp. per min.) is large relative to functional residual capacity, the net effect may be a normal average *time* N_2 molecules remain in the lungs.

DISCUSSION

Reproducibility

The procedure was repeated on five healthy subjects and six patients after a 10–20 minute period of N_2 re-equilibration, breathing air. In five cases, the first and second records were analyzed by different persons, and in six cases by the same person. As shown in Table III, the rapidity of N_2 clearance and the extent of uneven ventilation were similar on both tests. Statistical analysis showed (*1*) the variation between subjects greatly exceeded the variation between duplicates ($F = 14.9$), and (*2*) large correlation coefficients between duplicate values,

TABLE III

Duplicate N_2 clearance measurements

Subject	Average actual breath number		Pulmonary N_2 clearance delay (%)	
	1	2	1	2
M. N.	7.6	7.7	0	5
H. B.	7.1	8.0	13	13
R. W.	5.9	6.8	24	16
M. G.	10.2	9.9	12	33
J. G.	24.9	21.0	50	89
G. R.	11.0	12.7	70	45
H. S.	12.1	14.8	47	43
J. B.	12.5	10.3	87	51
L. W.	16.1	12.0	108	47
G. Y.	42.5	26.0	154	146
N. R.	87.5	75.5	215	164
Mean	21.6	18.6	70.9	59.3

being 0.98 for average actual breath numbers and 0.90 for pulmonary N_2 clearance delay values. More data are required to establish the error of a single determination of pulmonary clearance delay, particularly in patients, since the absolute difference between duplicate measurements appears to vary with the magnitude thereof.

Limitations

The accuracy of the mean expired N_2 concentrations was checked by the simultaneous measurement of FRC (*1*) by the Darling method and (*2*) by the values ($V_{L_1} + V_{L_2} + V_{L_3}$) obtained from tidal volume and mean N_2 concentration (Tables I and II). Apparently there was no important systematic error, since the best fitting straight line of regression of FRC_y (from mean expired N_2 concentration) on FRC_x (Darling) was

$$FRC_y = 9 + 0.962\ (\pm\ 0.089)\ FRC_x.$$

However, in certain subjects, particularly the patients, considerable discrepancies occurred. In general, these should probably be attributed to errors in the values calculated from the N_2 clearance curve. The equations assume constancy of tidal, dead space, and lung volumes throughout the procedure. The errors introduced by respiratory changes, if they occur, vary in nature and degree with different types of curves, and also depend on whether the respiratory changes are systematic, such as a progressive increase in tidal volume, and the part of the clearance process in which they occur. Considerable changes in tidal volume have been observed during the first several minutes of O_2 inhalation, both in normal subjects and in patients. These were probably related to (*1*) changes in respiratory resistance occurring when the O_2 circuit was attached, and (*2*) reduction of reflex respiratory stimulation in anoxemic subjects by the increased arterial pO_2 associated with O_2 inhalation.[7] In the two healthy subjects showing single phase records (M. N. and L. M.) progressive increases of tidal volume, occurring in the N_2 clearance period, probably obscured some degree of uneven ventilation. One healthy elderly subject showed such marked cyclic changes in tidal volume that the resulting record could not be analyzed. Progressive decreases in tidal volume of the emphysematous subjects probably caused an over-estimate of the extent of uneven ventilation; the prolonged average periods, two to four minutes, during which the N_2 molecules remained in their lungs, were also associated with a reduced minute volume of alveolar ventilation during O_2 inhalation by these anoxemic patients (average resting arterial O_2 saturation was 88.7%).

As noted before, the constant correction factor used for N_2 eliminated from blood and tissues is somewhat inaccurate. However, it has been used because its error is exceeded by that of determining the mean expired N_2 concentration, and the exact correction, at present unknown, must await knowledge of the relative blood flow through the various ventilatory regions of the lungs. The limited accuracy of our method at low N_2 concentrations precluded the attempt to obtain information about blood and tissue N_2 elimination by a more prolonged procedure. The multiphasic shape of the corrected clearance curves could be possibly attributed to an underestimate of the correction factor in only four of 36 cases.

However, we believe the magnitudes of uneven ventilation indicated by the values of pulmonary N_2 clearance delay are valid in general. Twenty-seven of these subjects (nine normals and 18 patients) were also examined with another method of estimating the magnitude of uneven

[7] A preliminary period of breathing compressed air from a demand regulator similar to that used for O_2 delivery, and the use of another N_2-free gas, such as 80% helium—20% O_2, might reduce the inconstancy of tidal volume.

ventilation, which is based on the variation in N_2 concentration of alveolar gas expired after a single maximal inspiration of O_2 (18, 19). Although this latter method is, at least theoretically, not a perfect and direct index, it is independent of the sources of error affecting the analysis of the N_2 clearance curves. There was general agreement between the two methods, as indicated by a highly significant, and fairly strong, correlation ($r = +0.76$).

One can obtain the total effective tidal volume ($V_T - V_D$) from analysis of the clearance curves. This obviously permits a measurement of respiratory dead space, which, if of unreasonable magnitude, indicates errors arising from (*1*) inconstancy of the respiratory volumes during the clearance process and/or (*2*) inaccuracies in the graphic analysis procedure. The average dead space value, corrected for apparatus dead space, for all male subjects (Tables I and II) was 137 ml., and for all female subjects, 127 ml. These are quite reasonable values and support the validity of the method. However, we do not recommend this method for measurement of respiratory dead space. It is unduly liable to errors arising from slight variations in determination of slope and position of the function describing the ventilation of hyperventilated areas. The direct method described previously (22, 23) is superior.

Comparison of methods

The "single breath" method for estimating uniformity of alveolar ventilation, noted above, requires only a single deep breath for its execution and only a few minutes for measurement. Theoretically, however, the variation in N_2 concentration of expired alveolar gas depends both on uneven end-inspiratory distribution of inspired O_2, and also on the change during expiration of proportion of gas expired from various lung areas (18, 19). The correlation noted above between the "single breath" results and the N_2 clearance analyses supports the general validity of the "single breath" test. Comparison was made to see if those patients judged to be abnormal by the single breath test were also abnormal by the values of pulmonary clearance delay. The appropriate mean plus 2 S.D. was used as the upper limit of normality. The results were identical in 15 of 18 patients, including all the cases of asthma, emphysema, bronchiectasis, sarcoid, and pulmonary fibrosis. The discrepancies occurred in two cases of congestive heart failure and one subject after pneumonectomy. The latter, however, probably merely indicates the inadequacy of the current data, based on only seven elderly subjects, to define clearly the normal variation of clearance delay values. Therefore, it currently appears that an abnormal "single breath" test is a reliable sign of abnormally uneven ventilation in subjects with this type of primary pulmonary disease. Further validation of the single breath test would be desirable in other types of patients, such as those with congestive heart failure, and in testing of uneven ventilation before and after therapy. Of the two tests, the single breath test, being rapidly and easily executed and measured, is preferable for the routine clinical detection of abnormally uneven ventilation.

However, the analysis of the pulmonary N_2 clearance curves yields much more information. First, the value of pulmonary N_2 clearance delay is a quantitative measurement of the extent of uneven ventilation. Its theoretical advantages recommend it for investigative use, particularly in the measurement of changes in the extent of uneven ventilation, such as might occur with therapy. Second, a rather comprehensive and quantitative picture of the volume and time characteristics of alveolar ventilation are obtained, both for the lungs as a whole and for its ventilatory subdivisions. Alveolar ventilation is the first step in the process of pulmonary gas-blood exchange. Further studies of alveolar diffusion and perfusion are possible when alveolar ventilation can be independently described.

Accuracy of the Darling method for measurement of functional residual capacity

This method requires knowledge of the change in the mean N_2 concentration of alveolar gas occurring in a seven minute period of O_2 inhalation. An estimate of the final alveolar N_2 concentration is obtained by analysis of the terminal portion of a forceful expiration, delivered at the end of seven minutes. The question whether such a sample accurately represents the mean alveolar N_2 concentration, particularly in subjects with uneven and delayed alveolar ventilation, has

been considered both by Darling and others (25). In the subjects above, continuous analysis of this forceful expiration was made. The N_2 concentration of the terminal part was abnormally high (>2%) in the five cases with emphysema and the two cases of severe asthma (average 4.6%). However, alveolar gas expired earlier in the same expirations had lower N_2 concentrations. The N_2 concentration of the unexpired residual gas was of course not directly measurable.

The *mean* alveolar N_2 was estimated from the N_2 clearance curves of the seven cases noted above as follows. The fractional changes of alveolar N_2 concentration per breath in the various lung areas are given by w_1, w_2, and w_3. The initial N_2 concentration in all areas was about 80%. After seven minutes, the N_2 concentration in the more rapidly ventilated phases 2 and 3 is practically equal to the inspired concentration. The alveolar N_2 concentration in phase 1 equals $(80 - F_I)\ w_1^n + F_I$, where n equals resp. per min. $\times$ 7. Since the fractional values of the total volume occupied by the various phases are known, the mean alveolar concentration is readily calculated.[8]

Mean values of the seven cases were, for the forced expiration alveolar sample, 4.6%; for the calculated *mean* alveolar N_2 concentration, 8.2%; for the alveolar concentration of phase 1, 11.0%. That is, the alveolar sample in subjects such as these with small expiratory reserve/residual capacity ratios, comes predominantly from relatively hyperventilated areas. Calculations using the expired alveolar sample concentration, therefore, yielded FRC values which, in these cases, were about 5% too small. Practically, this is unimportant. In fact this demonstration that the error is small in these extreme cases increases our confidence in the Darling method. In clinical usage, calculations based on the expired alveolar sample concentration are satisfactory. As a method for measurement of functional residual capacity, the Darling method is preferable to the analysis of pulmonary N_2 clearance curves, on the basis of apparatus simplicity, and rapidity and accuracy of measurement.

[8] The Darling FRC values given for these seven subjects in Table II were calculated by using this value rather than the N_2 concentration of the alveolar sample.

Other data

Robertson, Siri, and Jones have recently reported findings similar to those given in Tables I and II, based on multiphasic analysis of pulmonary N_2 elimination rates (12). However, we prefer to treat alveolar ventilation as a discontinuous rather than as a continuous process, as they have done. This permits the derivation of the pulmonary N_2 clearance delay, which is determined by the extent of uneven ventilation. Robertson and associates use a measure "E" of the efficiency of ventilation, which is dependent not only on the extent of uneven ventilation, but also on the dead space/tidal volume ratio, and, with certain methods of calculation, the respiratory frequency.

Bateman, in a recent study of pulmonary N_2 clearance of normal subjects, concluded that the behavior of the normal lung is compatible with a single chamber model (10). This is in contrast with data obtained by continuous analysis reported here and by Robertson, Siri, and Jones (12). However, one of several requirements upon which experiments were rejected or accepted was the "definiteness of linear relationship between y and n"; that is, the definiteness with which the clearance process could be described by a single value of w. As Robertson points out, analysis of multiphasic pulmonary clearance or equilibration curves with equations containing only one exponential term (4, 6, 10) is inadequate and may lead to ambiguous or at times incorrect interpretation of the nature of ventilatory inefficiency (23, 26). Bates and Christie (6) have achieved a clear cut separation of the ventilatory efficiency of certain types of subjects, quite in accordance with our results. However, their method, based on the 90% equilibration value, is at least theoretically incapable of demonstrating certain patterns of abnormally uneven ventilation which may exist in pulmonary diseases other than those reported.

INTERPRETATION

It is not clear why the lungs, either both or one (after pneumonectomy), with many anatomical subdivisions, appear to be ventilated as if they consisted of only two or three regions. Although this is of course possible, it seems more likely

that (a) technical inadequacies limit the resolution of the curves, and (b) the curves reflect patterns of the distribution of ventilation rates among the many regions. The measured curves were satisfactorily described in terms of regional inequalities of ventilation. However, we do not consider that this establishes the total or partial degree to which uneven ventilation is regional in nature. The possible mechanisms of uneven ventilation, which have been discussed elsewhere (19), and the spatial locations involved require further study.

SUMMARY

Measurements were made on successive breaths, during the elimination of pulmonary N_2 while breathing 99.6% O_2, of (*a*) the mean expired N_2 concentration, (*b*) number of breaths and (*c*) tidal volume. Equations with which the data have been described and analyzed are presented. The equations assume that uneven alveolar ventilation is of a regional nature.

The lungs, both in healthy young and old subjects and in patients with cardiorespiratory disease, are, in varying degrees, ventilated unevenly, *i.e.* certain regions appear to be ventilated more rapidly than other regions. The volumes and respective ventilation rates of these several regions can be determined, as well as the volume and average ventilation rate of the lungs as a whole. The extent of uneven ventilation can be measured quantitatively by the resulting delay in pulmonary N_2 clearance. This is determined relative to the rate of N_2 clearance which the subject would have achieved, breathing in the same manner, if alveolar ventilation had been uniform throughout the lungs. This measurement is uninfluenced by the magnitude of the respiratory dead space. In effect, a control and test procedure are done simultaneously for each subject.

In 10 healthy young adults, breathing quietly in the semi-reclining position, N_2 molecules remained in the lungs an average of 7.6 breaths or 36 seconds. This was 20% longer than if ventilation had been uniform. In seven elderly subjects N_2 molecules remained an average of 11.1 breaths, or 41 seconds. The increase in the average number of breaths was due to a smaller effective ventilation per breath and to a greater extent of uneven ventilation.

Abnormally uneven ventilation was found in almost all of 19 patients with cardiorespiratory diseases, including asthma, emphysema, congestive heart failure and others. The delaying effect of uneven ventilation can be overcome if the total minute volume of alveolar ventilation is large in relation to the functional residual capacity.

Twenty-seven of the subjects were also examined by another method of estimating the magnitude of uneven ventilation, which is based on the variation in N_2 concentration of alveolar gas expired after a single maximal inspiration of O_2. There was general agreement between the two methods, the relative utility of which is discussed.

The functional residual capacity, measured from the pulmonary N_2 clearance curves, agreed satisfactorily with simultaneous measurements by the method of Darling and coworkers. Analysis of the N_2 clearance curves permitted an estimate of the error of the Darling method. This amounted to about a 5% systematic underestimate in emphysematous subjects, resulting from the failure of the terminal alveolar gas sample to represent accurately the mean alveolar N_2 concentration.

Although the method presented permits an improved description of alveolar ventilation, a final interpretation of such data must await further study of the spatial distribution and the controlling mechanisms involved in alveolar ventilation.

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REFERENCES

1. Fowler, W. S., Cornish, E. R., Jr., and Kety, S. S., Measurements of alveolar ventilatory components. Am. J. Med. Sc., 1950, **220**, 112.
2. Fowler, W. S., Cornish, E. R., Jr., and Kety, S. S., Measurement of uneven alveolar ventilation. Am. J. Physiol., 1950, **163**, 711.
3. Fowler, W. S., Intrapulmonary gas mixing, *in:* Methods in Medical Research, edited by Comroe, J. H. Year Book Publishers, Chicago, 1950, Vol. II., p. 226.
4. Birath, G., Lung volume and ventilation efficiency. Acta med. Scandinav., 1944, Suppl. **154**.

5. Meneely, G. R., and Kaltreider, N. L., The volume of the lung determined by helium dilution. Description of the method and comparison with other procedures. J. Clin. Invest., 1949, **28**, 129.
6. Bates, D. V., and Christie, R. V., Intrapulmonary mixing of helium in health and in emphysema. Clin. Sc., 1950, **9**, 17.
7. Briscoe, W. A., Intrapulmonary mixing of helium in normal and emphysematous subjects. Abstracts, 18th International Physiological Congress, Copenhagen, August, 1950.
8. Englehardt, A., Uber den Verlauf der Entluftung der Lunge bei reiner Sauerstoffatmung. Ztschr. f. Biol., 1939, **99**, 596.
9. Darling, R. C., Cournand, A., and Richards, D. W., Jr., Studies on the intrapulmonary mixture of gases. V. Forms of inadequate ventilation in normal and emphysematous lungs analyzed by means of breathing pure oxygen. J. Clin. Invest., 1944, **23**, 55.
10. Bateman, J. B., Studies of lung volume and intrapulmonary mixing. Nitrogen clearance curves: apparent respiratory dead space and its significance. J. Appl. Physiol., 1950, **3**, 143.
11. Jones, H. B., Respiratory system: Nitrogen elimination, *in:* Glasser, I., Medical Physics. Year Book Publishers, Chicago, 1950, Vol. II, p. 855.
12. Robertson, J. S., Siri, W. E., and Jones, H. B., Lung ventilation patterns determined by analysis of nitrogen elimination rates; use of the mass spectrometer as a continuous gas analyzer. J. Clin. Invest., 1950, **29**, 577.
13. Miller, F., Hemingway, A., Varco, R. L., and Nier, A. O. C., Alveolar ventilation studies using the mass spectrometer. Proc. Soc. Exper. Biol. & Med., 1950, **74**, 13.
14. Boothby, W. M., Lundin, G., and Helmholz, H. F., Jr., A gaseous nitrogen elimination test to determine pulmonary efficiency. Proc. Soc. Exper. Biol. & Med., 1948, **67**, 558.
15. Wolfe, W. A., and Carlson, L. D., Studies of pulmonary capacity and mixing with the nitrogen meter. J. Clin. Invest., 1950, **29**, 1568.
16. Standardization of definitions and symbols in respiratory physiology. Federation Proc., 1950, **9**, 602.
17. Lilly, J. C., Mixing of gases within respiratory system with a new type nitrogen meter. Am. J. Physiol., 1950, **161**, 342.
18. Comroe, J. H., Jr., and Fowler, W. S., Lung function studies. VI. Detection of uneven alveolar ventilation during a single breath of oxygen. Am. J. Med., 1951, **10**, 408.
19. Fowler, W. S., Lung function studies. III. Uneven pulmonary ventilation in normal subjects and in patients with pulmonary disease. J. Appl. Physiol., 1949, **2**, 283.
20. Copperman, R., Personal communication.
21. Cournand, A., Yarmush, I. G., and Riley, R. L., Influence of body size on gaseous nitrogen elimination during high oxygen breathing. Proc. Soc. Exper. Biol. & Med., 1941, **48**, 280.
22. Fowler, W. S., Lung function studies. II. The respiratory dead space. Am. J. Physiol., 1948, **154**, 405.
23. Fowler, W. S., Lung function studies. V. Respiratory dead space in old age and in pulmonary emphysema. J. Clin. Invest., 1950, **29**, 1439.
24. Darling, R. C., Cournand, A., and Richards, D. W., Jr., Studies on intrapulmonary mixture of gases. III. An open circuit method for measuring residual air. J. Clin. Invest., 1940, **19**, 609.
25. Gilson, J. C., and Hugh-Jones, P., The measurement of total lung volume and breathing capacity Clin. Sc., 1949, **7**, 185.
26. Fowler, W. S., and Blakemore, W. S., Lung function studies. VII. The effect of pneumonectomy on respiratory dead space. J. Thoracic Surg., 1951, **21**, 433.

THE SOFFER TEST IN ESSENTIAL HYPERTENSION

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The hypertension occurring in association with adrenal cortical hyperfunction (Cushing's syndrome and congenital adrenal hyperplasia), the difficulty in establishing or maintaining experimental hypertension in the absence of the adrenal gland (1–3), and the amelioration of human "essential" hypertension by adrenalectomy (4) or the development of adrenal insufficiency (5) have led several investigators (6–8) to suggest that adrenal hyperfunction might contribute to the pathogenesis of human "essential" hypertension.

Attempts by a number of investigators to demonstrate this hyperfunction have been unsuccessful. Selye (9), Bruger, Rosenkrantz and Lowenstein (10), and Daughaday, Jaffe and Williams (11) have found the analysis of metabolic excretory products of the adrenal cortex (17-ketosteroids and 11-oxysteroids) in the urine of patients with hypertension to yield normal or low values. Careful study of carbohydrate metabolism in patients with hypertension (12) by means of the insulin tolerance test has revealed no abnormalities. Nitrogen balance is easily maintained in hypertensive subjects with a small protein intake (13). We and others (14) have found the blood eosinophil count normal in hypertension. Schroeder, Davies and Clark (15) have shown that the excretion of sodium in the sweat is normal in ordinary essential hypertension.

The renaissance of interest in the therapeutic efficacy of a low sodium regime in the treatment of hypertension has focused attention particularly on the electrolyte controlling properties of the adrenal cortex as a possible mechanism for adrenal contribution to the pathogenesis of hypertension. The plasma electrolyte abnormality (hypochloremic alkalosis) frequently seen in Cushing's syndrome is not seen in hypertension. Most observers have found no plasma electrolyte abnormality in hypertension although Selye (9) believes the ratio of sodium to chloride is high.

Soffer and his associates (16, 17) have pointed out that patients with Cushing's syndrome have an abnormal response to salt loading and the administration of desoxycorticosterone. His normal individuals, after a given salt load, retained more of the salt after the administration of desoxycorticosterone than they did without this drug. On the other hand, the patients with Cushing's syndrome showed an increased "diuresis" of the salt load after desoxycorticosterone. This observation has been confirmed by others (18, 19).

It is conceivable, then, that should patients with essential hypertension have adrenal cortical hyperfunction with respect to electrolyte metabolism they would exhibit this same abnormal response.

METHODS

The salt loading tests were carried out on a group of patients with hypertension following the exact regime outlined by Soffer. The patients were permitted no food during the morning of the test and no fluid after 7 p.m. the night before. At 6 a.m. on the morning of the studies a urine specimen was collected and discarded. The patient was then given 500 cc. of water to drink at one time, and all the urine voided was collected over a three hour period to 9 a.m. At 9 a.m., 200 cc. of 5% saline (171 milliequivalents of sodium and of chloride) were injected intravenously and the urine voided the next three hours was collected. Two days later the same procedure was repeated, except that at 10 p.m. on the previous evening the patient was injected with 10 mgm. of desoxycorticosterone acetate (DCA). The urine volume for each period was measured and sodium and chloride determinations were made on each specimen. Sodium was determined by flame photometry and chlorides according to the method of Van Slyke and Hiller (20).

The hypertensive patients used in this study were 10 ambulatory male patients chosen from the outpatient clinic according to the following criteria: a history in any way suggestive of congestive heart failure, abnormal urinary sediments, or elevated blood urea nitrogen were causes for exclusion. After selection, patients were placed on restricted activity in the hospital and blood pressure determined twice daily at 6 a.m. and 6 p.m. in the recumbent position for seven days. Any blood pres-

TABLE I

Subj.	Control				*After injection of 10 mgm. of DCA*				Excretion			
									Decrease		Increase	
	Urine vol. 6–9 a.m.	Urine vol. 9–12 a.m.	Injected Na Excreted Na_c	Injected Cl Excreted Cl_c	Urine vol. 6–9 a.m.	Urine vol. 9–12 a.m.	Injected Na Excreted Na_d	Injected Cl Excreted Cl_d	$100 \times \frac{(Na_c - Na_d)}{Na_c}$	$100 \times \frac{(Cl_c - Cl_d)}{Cl_c}$	$100 \times \frac{(Na_d - Na_c)}{Na_c}$	$100 \times \frac{(Cl_d - Cl_c)}{Cl_c}$
	cc.	cc.			cc.	cc.						
P. R. 1	275	675	19.8%	20.1%	150	700	19%	21.9%	4%	—	—	8.2%
P. R. 2	325	850	48.5	36	515	673	39.4	28.3	18.8	21.4	—	—
T. R. 1	275	200	8.2	5.2	240	150	−3.3	2.9	140	44	—	—
T. R. 2	335	195	8.8	10.3	208	130	5.3	7.4	39.8	28.2	—	—
V. A. 1	100	125	2	3	90	250	19.7	16.9			90	82
V. A. 2	49	245	23.3	26.4	74	130	4.3	7.9	82	70.1		
H. K.	250	275	16.9	15.6	76	140	8.1	7.0	52.1	55		
L. E.	254	462	35.2	35.2	190	470	22.4	22.5	36.4	36.3	—	—
B. E.	520	630	39.4	40.3	400	595	23.5	16.8	40.4	58.3	—	—
M. C.	510	310	19	3.7	440	185	4.2	1.7	77.8	54.0	—	—
H. N.	320	180	13.1	14.8	290	130	9.6	6.8	26.7	54	—	—
D. E.	215	315	21.3	23.8	490	169	−1.1	−6.3	105	126	—	—
T. S.	195	253	11.1	10.2	166	213	8.8	11.1	20.7			8.8

sure reading below 160/105 mm. Hg during this period or during the subsequent study period disqualified the patient. The patients were also placed on diets containing at least 5 gm. of salt daily for four days prior to the test. The age distribution of these patients varied from 33 to 65 with a mean age of 51.5 years.

RESULTS

In Table I are charted the data from 13 complete tests on ten male patients with essential hypertension. For convenience and ease of comparison with Soffer's data, these data are charted in the same fashion. It must be realized that the figures in the last two columns do not represent absolute decrease or increase of sodium or chloride excretion in milliequivalents but only percentage of change between the control and the DCA period. Nevertheless, they effectively demonstrate the retention or diuresis of sodium and chloride in a given patient. In view of the fact that the test only assesses the excretion of sodium and chloride for the first three hours after the administration of the load, it is doubtful that plotting excretion changes in milliequivalents would be of any more value. Despite its obvious shortcomings, the fact that this test clearly demonstrates the electrolyte abnormality in most cases of Cushing's syndrome makes its use seem worthwhile in clinical evaluation of a possible adrenal controlled electrolyte abnormality in essential hypertension.

It is evident that nine of the ten patients responded with a clear-cut retention of sodium and chloride after DCA which is the normal response. One patient (V. A. 1) showed a very low control excretion of sodium and chloride which was responsible for an apparent salt diuresis. When this test was repeated with more normal control excretion he showed the normal retention effect with DCA.

SUMMARY AND CONCLUSIONS

1. The Soffer salt tolerance test was carried out 13 times in ten selected patients with hypertension.

2. Nine of the ten patients showed a retention of chloride and all ten of the patients a retention of sodium after salt loading and DCA. This is a normal response.

3. These data fail to demonstrate an electrolyte abnormality in essential hypertension similar to that found in patients with adrenal cortical hyperfunction of the Cushing type.

REFERENCES

1. Goldblatt, H., Studies on experimental hypertension; pathogenesis of experimental hypertension due to renal ischemia. Ann. Int. Med., 1937, **11**, 69.
2. Page, I. H., The effect of bilateral adrenalectomy on arterial blood pressure of dogs with experimental hypertension. Am. J. Physiol., 1938, **122**, 352.
3. Collins, D. A., and Wood, E. H., Experimental renal hypertension and adrenalectomy. Am. J. Physiol., 1938, **123**, 224.
4. Green, D. M., Nelson, J. N., and Dodds, G. A., Effects of adrenal resection on hypertension and diabetes. Federation Proc., 1949, **8**, 60.

5. Perera, G. A., The relationship of the adrenal cortex to hypertension; observations on the effect of hypoadrenalism on a patient with hypertensive vascular disease. J.A.M.A., 1945, **129**, 537.
6. Perera, G. A., The adrenal cortex and hypertension. Bull. New York Acad. Med., 1950, **26**, 75.
7. Alexander, A. S., and Young, R. W., Hypertension treated by irradiation of the adrenals; with report of 23 cases. New Orleans M. & S. J., 1949, **101**, 536.
8. Crile, G. W., Indications and contraindications for denervation of the adrenal glands. Ann. Surg., 1934, **100**, 667.
9. Selye, F. L., Biochemical changes in hypertension. Canad. M. A. J., 1947, **57**, 325.
10. Bruger, M., Rosenkrantz, J. A., and Lowenstein, B. E., Studies on morphology of adrenal cortex and on excretion of 17-ketosteroids in hypertensive patients. Am. J. M. Sc., 1944, **208**, 212.
11. Daughaday, W. H., Jaffe, H., and Williams, R. H., Adrenal cortical hormone excretion in endocrine and non-endocrine disease as measured by chemical assay. J. Clin. Endocrinol., 1948, **8**, 244.
12. Mirsky, I. A., Kaplan, S. M., Podore, C. J., and Broh-Kahn, R. H., The insulin tolerance test in patients with essential hypertension. J. Clin. Invest., 1950, **29**, 297.
13. Watkin, D. M., Froeb, H. F., Hatch, F. T., and Gutman, A. B., Effects of diet in essential hypertension. II. Results with unmodified Kempner rice diet in fifty hospitalized patients. Am. J. Med., 1950, **9**, 441.
14. Laragh, J. H., and Almy, T. P., Changes in circulating eosinophils in man following epinephrine, insulin, and surgical operations. Proc. Soc. Exper. Biol. & Med., 1948, **69**, 499.
15. Schroeder, H. A., Davies, D. F., and Clark, H. E., A syndrome of hypertension, obesity, menstrual irregularities, and evidence of adrenal cortical hyperfunction. Proc. Central Soc. Clin. Research, 1949, **22**, 73; J. Lab. & Clin. Med., 1949, **34**, 1746.
16. Soffer, L. J., Lesnick, G., Sorkin, S. Z., Sobatka, H. H., and Jacobs, M., The utilization of intravenously injected salt in normals and in patients with Cushing's syndrome before and after the administration of desoxycorticosterone acetate. J. Clin. Invest., 1944, **23**, 51.
17. Soffer, L. J., Gabrilove, J. L., and Jacobs, M. D., Further studies with the salt tolerance test in normal individuals and in patients with adrenal cortical hyperfunction. J. Clin. Invest., 1949, **28**, 1091.
18. Thorn, G. W., Merrill, J. P., Smith, S., Roche, M., and Frawley, T. F., Clinical studies with ACTH and cortisone in renal disease. Arch. Int. Med., 1950, **86**, 319.
19. Cargill, W. H., and St. John, H. A., Desoxycorticosterone and the urinary excretion of sodium chloride. Am. J. Med., 1950, **9**, 395.
20. Van Slyke, D. D., and Hiller, A., Application of Sendroy's iodometric chloride titration to protein-containing fluids. J. Biol. Chem., 1947, **167**, 107.

THE NORMAL ANTIDIURETIC MECHANISM IN MAN AND DOG; ITS REGULATION BY EXTRACELLULAR FLUID TONICITY [1, 2]

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The work of Verney (1, 2) has clearly demonstrated that the antidiuretic mechanism of the posterior pituitary is sensitive to changes in total effective solute concentration of the extracellular fluid. He postulates osmoreceptors which cause out-pouring of antidiuretic hormone of the posterior pituitary in response to an increased concentration of solute in the serum and inhibition of antidiuretic hormone production with dilution of the extracellular fluids.

Others have demonstrated antidiuretic activity in the urine or serum of rats (3, 4), cats (5) and dogs (6–8) following dehydration or after infusions of hypertonic saline solutions. Following water administration or hypophysectomy no antidiuretic activity is present in urine or serum.

The purpose of this paper is to further describe the mechanism in normal man and dogs which acts to preserve the total solute concentration of the serum within narrow limits, and to present the methods and form of presentation which will be used in an accompanying paper (9).

METHODS AND PROCEDURE

The data were obtained from ten separate studies on two normal male human adults and from 12 studies on three normal unanesthetized trained female dogs. Urine and serum collections were made before and at hourly intervals following administration of 670 to 3,000 ml. of water to the human subjects and 300 to 400 ml. of water by stomach tube to the dogs. Urine collections from the dogs were made by catheterization.

Serum sodium determinations were done in duplicate by the method of Butler and Tuthill (10). Serum and urine total solute concentrations were determined by the freezing point method utilizing an electrical resistance thermometer apparatus described by Crawford and Nicosia (11). The apparatus was standardized with sodium chloride solutions of known concentration. The assumption was made, for convenience of expression, that the sodium chloride was totally dissociated and the results were expressed as milliosmols per liter. More correctly, total solute concentration should be expressed as the concentration in millimols of sodium chloride which produces a depression of the freezing point equal to that produced by the test material. This expression is simply obtained by dividing the reported concentration in milliosmols by two. All sera were measured without dilution while urines were diluted, if necessary, into the range of 100–300 mOsm/L. This dilution, by changing the activity coefficient of certain solutes, introduces a slight but systematic error which does not affect our interpretations.

The serum assay for antidiuretic activity utilized four posthypophysectomized rats,[4] individually weighing approximately 200 gms., in each determination. The animals were given 8 ml. of water per 100 square centimeters of surface area ($11.23 \times \text{weight}^{2/3}$) by stomach tube following 12 hours fasting. Two-fifths of the water was administered initially and one-fifth at hourly intervals thereafter. Saline or serum inactivated by 48 hours storage was injected intraperitoneally at the start of the third hour of hydration. One to 2 ml. of fresh test serum, within 30 minutes of venipuncture, were injected intraperitoneally into each rat at the start of the fourth hour. The rats were kept in metabolism cages over funnels and all urine was collected during the third and fourth hours. Total solute concentration of the urines was then determined and the mean increase in concentration of the rats' urine during the hour following injection of the test material as compared with the preceding control hour was taken as the antidiuretic index.[5]

As seen in Table I, when only saline was injected there was no essential change in urine concentration during the third and fourth hours of the test—Part A. Similar results were obtained when serum from a hydrated subject replaced the second saline injection—Part B. However, when serum obtained from a dehydrated subject replaced the second injection an increase in urine concentration occurred—Part C. Part D shows what was

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[2] Presented in part at the Forty-third Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., April 30, 1951.

[3] National Research Council Fellow in the Medical Sciences, 1949–1951.

[4] We are indebted to Dr. Charles Read for generously supplying us with operated rats.

[5] The idea of using change in urine concentration as the index of antidiuretic activity was suggested by Dr. J. D. Crawford who had utilized it prior to us.

TABLE I

Examples of assays for antidiuretic activity in serum

	Hour	Rat 1	Rat 2	Rat 3	Rat 4	Material assayed
		Urine concentration (mOsm/L)				
A	3	119	110	112	74	1 ml. saline
	4	112	86	96	70	1 ml. saline
	Antidiuretic index = −13 (p = 0.43)					
B	3	178	122	165	85	1 ml. serum (inactive)
	4	143	133	138	97	1 ml. serum (hydrated dog)
	Antidiuretic index = − 4 (p = 0.43)					
C	3	153	136	103	46	1 ml. saline
	4	223	285	324	77	1 ml. serum (dehydrated dog)
	Antidiuretic index = +118 (p = 0.03)					
D	3	118	75	65	200	1 ml. saline
	4	258	174	90	250	0.5 milliunits Pitressin
	Antidiuretic index = +79 (p = 0.03)					

observed when 0.5 milliunit of Pitressin was substituted for the second saline injection.

The assays were treated statistically as follows. There were 30 control assays done in which no antidiuretic material was injected, giving a total of 115 observations of the response of individual rats. The mean change in urine solute concentration per rat was + 3.1 mOsm/L with a standard deviation of 47. In these 30 control assays the mean number of rats per assay showing a change in urine concentration greater than one standard deviation above the mean of the control series was 0.83 with a standard deviation of 0.87. Each test assay was then assessed to see whether the number of rats showing an increase of urine concentration greater than one standard deviation above the mean of the control series was significant. The value of "p" following each antidiuretic index gives the probability of its significance. Values of "p" of 0.03 or less were considered satisfactory confidence limits for this study.

No attempt was made to assign quantitative significance to the assay results. The conditions under which each assay was conducted were such as to make either a positive or a negative response meaningful. Thus no significance should be read into the relative magnitude of the antidiuretic indices reported. Whatever the magnitude of this index the accompanying "p" value is alone of importance. A "p" value of 0.03 or less is considered a "positive" assay while a larger value for "p" indicates no significant difference from the control group, and hence, a "negative" assay.

RESULTS

Table II contains data taken from a representative experiment on subject A. L. Similar representative data on A.R.M. and Dog B are recorded in Tables III and IV, respectively, and Figure 1. Total solute concentration of urine and serum is plotted on the ordinate with time on the abscissa. The rate of urine flow is shown at the bottom. Because two assays could not be done on the same day the dehydration actually is separated by several days from the hydration measurements but for purposes of charting these are combined. The results of several assays done on different occasions also appear in Figure 1.

Following the period of water deprivation there occurred a slight but significant rise of serum total solute concentration above the average normal for each species (314 mOsm/L for man and 331 mOsm/L for the dog, indicated by the broken line). At this time each was found to be excreting a urine of nearly maximal concentration for its species. Upon administration of water, there resulted a dilution of the serum accompanied by

TABLE II

A. L. Normal male adult

A. Dehydration periods

Time	Serum		Urine		Prep.	Assay
	Solute conc.	Na	Flow	Solute conc.		
	mOsm/L	*mEq/L*	*ml./min.*	*mOsm/L*		
4/4/50	310	141		1,020	18 hrs. no water	+38 (p = 0.20)
4/25/50	315	144		923	24 hrs. no water	No assay
6/21/50	316	137		1,150	24 hrs. no water	No assay

B. Hydration period—7/5/50

Hour						
0	309	144		1,020		
0.5 to 1. 670 ml. water ingested						
1			1.6	435		
2	304	141	3.3	126		−23 (p = 0.43)
3			0.8	600		
4			0.6	924		
5			0.6	900		
8	305	144	0.5	963		

Average normal human serum (water *ad lib*) = 314 mOsm/L (range 302–323); Na = 141 mEq/L (range 138–144).

a progressive dilution of the urine until concentrations distinctly hypotonic to the serum or extracellular fluid were achieved. The rate of urine flow at the bottom of each figure illustrates the water diuresis that occurred. After the administered water load had been excreted the serum and urine total solute concentrations again rose.

Furthermore, at the end of the period of water restriction it was possible to demonstrate antidiuretic activity in the serum obtained from the jugular veins of the dog. During the diuresis that followed water administration, no antidiuretic activity was demonstrated in the serum. In the two normal human subjects antidiuretic activity was not conclusively demonstrated in the serum.

As stated below no claims can be made for the specificity of the antidiuretic assay. That the posterior pituitary is involved in water excretion is common knowledge and that its integrity is essential for the mechanism under discussion is indicated by the data in Figure 2. The results on two occasions of water deprivation for 14 hours in a patient with posterior pituitary insufficiency are shown. The presence of posterior pituitary insufficiency was established by these data and the patient's ability to elaborate a concentrated urine (1040 mOsm/L) while receiving small amounts of posterior pituitary extract. In Figure 2 it is seen that in spite of severe dehydration as indicated by a very marked increase in serum total solute concentration (the serum sodium concentration rose to 173 mEq/L in the second period), this patient's urine remained persistently dilute. Similar, though less striking results, have been obtained on three other patients with clinical diabetes insipidus.

TABLE III

A. R. M. Normal male adult

A. Dehydration periods

Time	Serum		Urine		Prep.	Assay
	Solute conc.	Na	Flow	Solute conc.		
	mOsm/L	*mEq/L*	*ml./min.*	*mOsm/L*		
2/9/51	322	146		1,260	24 hrs. no water	+76 (p=0.09)
4/19/51	321	143		1,029	24 hrs. no water	−5 (p=0.43)

B. Hydration period—4/16/51

Hour 0	310	142	0.55	1,155	15 hrs. no water	
0.083 to 0.166 hours. 1,250 ml. water orally						
0.33			1.0	1,044		
1			4.2	194		
2	303	138	3.2	255		+5 (p=0.43)
3			1.5	531		
4			0.8	651		
5	308	138	0.7	900		

Average normal human serum (water *ad lib*) = 314 mOsm/L (range 302–323); Na = 141 mEq/L (range 138–144).

TABLE IV

Dog B, Normal

A. Dehydration periods

Time	Serum		Urine		Prep.	Assay
	Solute conc.	Na	Flow	Solute conc.		
	mOsm/L	*mEq/L*	*ml./min.*	*mOsm/L*		
5/9/50	331	154			24 hrs. no water	+102 (p=0.03)
10/6/50	329	143		1,860	36 hrs. no water	+118 (p=0.03)
12/6/50	360	154		2,320	48 hrs. no water	+64 (p=0.20)
3/5/51	340	150		2,200	48 hrs. no water	+53 (p=0.03)

B. Hydration period—12/4/50

Hour 0	338	147		1,800		
400 ml. water by stomach tube						
1			0.2	655		
2	318	144	2.2	140		+9 (p=0.43)
3			0.6	150		
4	330	147	0.2	655		

Average normal dog serum (water *ad lib*) = 331 mOsm/L (range 320–340); Na = 146 mEq/L (range 145–147).

DISCUSSION

According to current physiological concept, which is largely derived from the excellent studies of Verney and his associates, Figure 1 would be

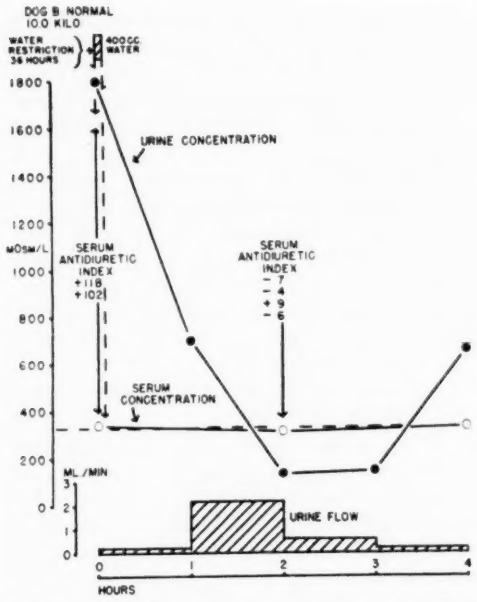

FIG. 1. THE NORMAL DIURETIC PATTERN IN THE DOG

Urine and serum total solute concentrations, expressed as milliosmols per liter, are plotted on the ordinate with time on the abscissa. The maximal urine concentration in the dog, about 2,000 mOsm/L, is considerably higher than in man. The average normal total solute concentration of the serum in this species, 331 mOsm/L, is indicated by the broken line. The slight visible changes in serum total solute concentration, from slightly elevated following water restriction to slightly depressed following water administration, serve again to emphasize the effectiveness of this mechanism for preserving a constant serum osmotic pressure in spite of large variations in fluid intake. The serum antidiuretic indices recorded indicate the presence of antidiuretic activity following water restriction when serum solute concentration is increased and urine maximally concentrated, and absence of antidiuretic activity when serum and urine are dilute following water ingestion. This is a composite figure showing data from more than one experiment.

explained as follows: As a result of restricted fluid intake and a continued insensible loss of water from the body there occurs a slight increase in total solute concentration of the serum and extracellular fluid. This stimulates, through "osmoreceptors," an increased activity of the supraopticohypophyseal system with outpouring of antidiuretic hormone. The antidiuretic hormone acts upon the renal tubule cells to produce an increased reabsorption of water from the glomerular filtrate (12) with resultant formation of a concentrated urine and conservation of body water. The dilution of the extracellular fluid which follows water administration inhibits posterior pituitary activity, creating a state of functional diabetes insipidus which permits diuresis of a dilute urine ridding the body of the excess water load. This cybernetic mechanism is thus seen to be ideally designed to preserve within narrow limits the total solute concentration of the extracellular fluid.

Verney has clearly shown that not all solutes are equally effective in stimulating the osmoreceptors (1). Thus urea was practically noneffective while NaCl and sucrose in equiosmolar

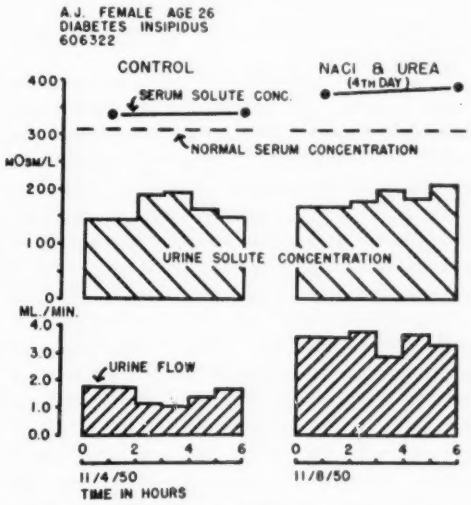

FIG. 2. THE EFFECTS ON SERUM AND URINE TOTAL SOLUTE CONCENTRATIONS OF 14 HOURS OF WATER RESTRICTION ON TWO OCCASIONS IN A PATIENT WITH DIABETES INSIPIDUS

On the second occasion sodium chloride 10 gms. and urea 15 gms. daily had been taken orally on the preceding three days to increase the excretory solute load. Total solute concentrations of serum and urine are plotted on the ordinate. Note that in spite of severe dehydration indicated by the markedly elevated serum total solute concentrations the urine remains very dilute indicating complete loss of ability on the part of the kidneys to conserve body water. However, with injections of small amounts of posterior pituitary extract this patient's kidneys were able to produce a urine of maximal concentration. The inability to concentrate the urine in the presence of dehydration can, therefore, be blamed on a failure of posterior pituitary activity.

concentrations produced prompt antidiuresis when infused into the internal carotid artery of a dog during a water diuresis. Apparently a substance which distributes itself readily through the intracellular compartment as well as the extracellular fluid fails to set up an osmotic gradient between the cell and its environment and hence fails as a stimulus to the "osmoreceptors." The freezing point determination of total solute concentration, of course, summates all the solutes present in the serum: those which exert an effective osmotic pressure on the osmoreceptors and those that do not. The short period of time over which the changes in serum concentration are observed and the parallel changes in serum sodium concentration obtained indicate that a true dilution is occurring and not just a reduced concentration of a single solute such as urea.

That a measurable dilution in the serum can be found following the enteral administration of as little as 350–400 ml. of water to a 10-kilogram dog or 670 ml. of water to a 75-kilogram adult human is somewhat surprising. Assuming total body water to be 60% of the body weight for both species, 350 ml. of water entirely absorbed from the gut and not excreted would result, in the dog, in an expected dilution of the serum of 5.8% and, in the human studied, 670 ml. would correspondingly cause a dilution of 1.5%. That changes of approximately this magnitude were obtained in spite of the presence of diuresis is probably to be explained by the delay in distribution of the ingested water throughout the total body water space. Schloerb and associates (13) found that following oral ingestion of deuterium oxide the rate of absorption from the gut is greater than the rate of distribution throughout the body and that some three hours were required before the serum level fell to the equilibrium concentration following the dose of D_2O used. Our findings are in accord with those of Baldes and Smirk (14), Margaria (15) and Lifson (16), who measured vapor pressure changes to obtain total solute concentration of serum.

It was hoped that an assay method for antidiuretic activity which measured directly the change in urine concentration would have advantages over the standard methods which depend upon the rate of elimination of a fixed water load. The latter method must depend upon the rate of solute excretion as well as upon the presence or absence of antidiuretic activity (17). Frequently no correlation was observed between urine volume and concentration, presumably due to variations in rate of solute excretion. Also it was hoped that by using post-hypophysectomized animals assay of substances (18–21) which exhibit their antidiuretic effects through release of endogenous antidiuretic hormone would be avoided. However, the post-hypophysectomized rats could always on dehydration exhibit the capacity to elaborate a more concentrated urine than the maximum response attained during the assays, so that this possibility is not excluded in the results.

This assay method proved to be very cumbersome, tedious and difficult to control.[6] Of a total of 72 assays one "false" positive assay (positive response following injections of material known to contain no antidiuretic activity) and many false negative assays were encountered. Though we have a reasonable degree of confidence in the assay results presented we wish to minimize the importance of the assay to our interpretation. No matter how reproducible and sensitive an assay of this type is, it will not prove that antidiuretic material found in the serum is of posterior pituitary origin.

The inability to demonstrate antidiuretic activity in the serum of the human subjects following water deprivation, although we were apparently successful in the dog, is probably attributable to two factors. First, the canine uncomplainingly tolerated longer periods of water deprivation than the humans. Secondly, serum for assay was obtained from the jugular veins of the dog but from the antecubital veins of the humans. The concentration of secretions of the posterior pituitary might be expected to be considerably higher in venous blood from the head than from the arm or leg (8).

The variability that occurs in endogenous posterior pituitary activity from no apparent function during water diuresis to maximal activity during dehydration makes it seem imperative that assays of antidiuretic material in body fluids be carried out under standard conditions of hydration in order to be interpretable. Otherwise, what might be

[6] It is expected that an improved form of this assay which can be applied quantitatively will subsequently be reported by Dr. J. D. Crawford.

concluded to be overactivity of the supraoptico-hypophyseal system in disease may simply be the normal response of this system to a disturbed state of hydration caused by the disease—*i.e.*, anorexia and nausea causing low fluid intake.

SUMMARY AND CONCLUSIONS

It is shown that in normal man and dog water deprivation causes a slight rise in serum total solute concentration associated with maximal concentration of the urine. Following water administration there occurs a slight but measurable dilution of the serum. With this the urine becomes progressively more dilute reaching levels distinctly hypotonic to the extracellular fluid. When the administered water load is excreted serum and urine total solute concentrations again rise.

Furthermore, in the dog, the presence of antidiuretic activity in the serum was demonstrated at the end of the period of water deprivation, while no activity was demonstrable during the diuresis that followed water administration. The role of the posterior pituitary in this mechanism is indicated by persistence of dilute urine during dehydration in spite of very marked hypertonicity of the serum in a patient with posterior pituitary insufficiency. Thus it is seen that this cybernetic mechanism is ideally designed to preserve within narrow limits the total effective solute concentration of the serum.

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REFERENCES

1. Verney, E. B., Absorption and excretion of water: antidiuretic hormone. Lancet, 1946, **2**, 781.
2. Verney, E. B., Antidiuretic hormone and the factors which determine its release. Proc. Roy. Soc. London, Series B, 1947, **135**, 25.
3. Gilman, A., and Goodman, L., The secretory response of the posterior pituitary to the need for water conservation. J. Physiol., 1937, **90**, 113.
4. Boylston, G. A., and Ivy, A. C., An antidiuretic substance present in the urine of dehydrated rats. Proc. Soc. Exper. Biol. & Med., 1938, **38**, 644.
5. Ingram, W. R., Ladd, L., and Benbow, J. T., The excretion of antidiuretic substance and its relation to the hypothalamico-hypophyseal system in cats. Am. J. Physiol., 1939, **127**, 544.
6. Hare, K., Hickey, R. C., and Hare, R., The renal excretion of an antidiuretic substance by the dog. Am. J. Physiol., 1941, **134**, 240.
7. Hickey, R. C., and Hare, K., The renal excretion of chloride and water in diabetes insipidus. J. Clin. Invest., 1944, **23**, 768.
8. Ames, R. G., Moore, D. H., and van Dyke, H. B., The excretion of posterior pituitary antidiuretic hormone in the urine and its detection in the blood. Endocrinology, 1950, **46**, 215.
9. Leaf, A., and Mamby, A. R., An antidiuretic mechanism not regulated by extracellular fluid tonicity. J. Clin. Invest., 1952, **31**, 60.
10. Butler, A. M., and Tuthill, E., An application of the uranyl zinc acetate method for determination of sodium in biological material. J. Biol. Chem., 1931, **93**, 171.
11. Crawford, J. D., and Nicosia, A. D., A method for determination of freezing points of small quantities of biological fluids. To be published.
12. Smith, H. W., The Kidney. Structure and Function in Health and Disease. Oxford University Press, New York, 1951, p. 257.
13. Schloerb, P. R., Friis-Hansen, B. J., Edelman, I. S., Solomon, A. K., and Moore, F. D., The measurement of total body water in the human subject by deuterium oxide dilution. J. Clin. Invest., 1950, **29**, 1296.
14. Baldes, E. J., and Smirk, F. H., The effect of water drinking, mineral starvation and salt administration on the total osmotic pressure of the blood in man, chiefly in relation to the problems of water absorption and water diuresis. J. Physiol., 1934, **82**, 62.
15. Margaria, R., The vapour pressure of normal human blood. J. Physiol., 1930, **70**, 417.
16. Lifson, N., Note on the total osmotic activity of human plasma or serum. J. Biol. Chem., 1944, **152**, 659.
17. Crawford, J. D., Schoen, E. J., and Nicosia, A. D., Factors determining maximum renal water excretion capacity. Proc. Soc. for Ped. Research, May, 1951.
18. de Bodo, R. C., The antidiuretic action of morphine, and its mechanism. J. Pharmacol. & Exper. Therap., 1944, **82**, 74.
19. de Bodo, R. C., and Prescott, K. F., The antidiuretic action of barbiturates (phenobarbital, amytal, pentobarbital) and the mechanism involved in this action. J. Pharmacol. & Exper. Therap., 1945, **85**, 222.
20. Pickford, M., Control of the secretion of antidiuretic hormone from the pars nervosa of the pituitary gland. Physiol. Rev., 1945, **25**, 573.
21. Shorr, E., Baez, S., Zweifach, B. W., Payne, M. A., Mazur, A., and Metz, D. B., The antidiuretic action of the hepatic vasodepressor ferritin (VDM) and its occurrence in conditions associated with antidiuresis in man. Tr. A. Am. Physicians, 1950, **63**, 39.

AN ANTIDIURETIC MECHANISM NOT REGULATED BY EXTRACELLULAR FLUID TONICITY [1, 2]

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In the preceding paper (1) we have described the antidiuretic mechanism in normal man and dogs which preserves within narrow limits the total effective solute concentration of the extracellular fluid. The increase in serum solute concentration that occurs with water deprivation stimulates outpouring of antidiuretic hormone which leads to a concentrated urine and renal conservation of body water. The dilution of the extracellular fluid that follows water administration suppresses activity of the supraopticohypophyseal system permitting water diuresis and renal excretion of the administered water load. This sequence of events was first recognized and demonstrated by Verney (2, 3).

If the above is accepted as the normal physiology, then the common clinical observation of sustained low serum sodium concentrations in patients who have no apparent intrinsic renal disease needs explanation. As sodium is the major osmotically active constituent of the extracellular fluid it might be expected that total solute concentration would be low if sodium were markedly reduced. But a dilute extracellular fluid would cause inhibition of antidiuretic hormone production with resultant water diuresis and return of serum sodium and total solute concentration to the normal level. Why does this not occur?

METHODS

Sodium determinations in serum and total solute concentrations of serum and urine were obtained as described (1). Urine sodiums were done with an internal standard flame photometer (4). The assays for serum antidiuretic activity were carried out as described (1). The "p" value following each assay gives the probability of its significance (1).

The subjects for this study were: (*1*) patients on the medical wards of the Massachusetts General Hospital including four patients with Addison's disease on whom 19 tests of water excretion were conducted and eight patients with congestive heart failure on whom 13 studies were performed; (*2*) three mongrel female dogs. The dogs were first depleted of extracellular fluid electrolyte by the intraperitoneal dialysis technique of Darrow and Yannet (5) and tested 24 hours later for their ability to excrete a water load. Urine collections were obtained from the dogs by catheterization. These same animals were then tested for their ability to excrete water following repeated small injections of Pitressin Tannate in Oil (Parke, Davis and Co.) 1.0 to 2.0 units twice daily.

RESULTS

A. In Addison's disease

Table I and Figure 1 show data obtained from a female patient whose Addison's disease was inadequately treated with 5 mgs. of desoxycorticosterone glucoside intramuscularly daily. It is clear that the low serum total solute concentration

TABLE I

(6/2/50) E. S. Female. Addison's disease receiving 5 mgs. DOCG daily

Time (*hours*)	Serum		Urine		Assay
	Na (*mEq/L*)	Total solutes (*mOsm/L*)	Flow (*ml./min.*)	Total solutes (*mOsm/L*)	
0				510	
1.5	125	281	0.5	456	
1.5–2.0	500 ml. water orally				
3.0	123	277	1.8	444	
4.0	124	262	0.8	552	+167 ($p < 0.03$)
5.0	121	260	0.6	660	
6.0	118	270	1.0	486	
7.5			0.9	564	
10.0	120	262	1.0	417	
12.5			1.6	369	
14.5			0.8	435	
19.0			1.3	288	
22.0			0.7	495	
24	125	279			

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[2] Presented in part at the Forty-third Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., April 30, 1951.

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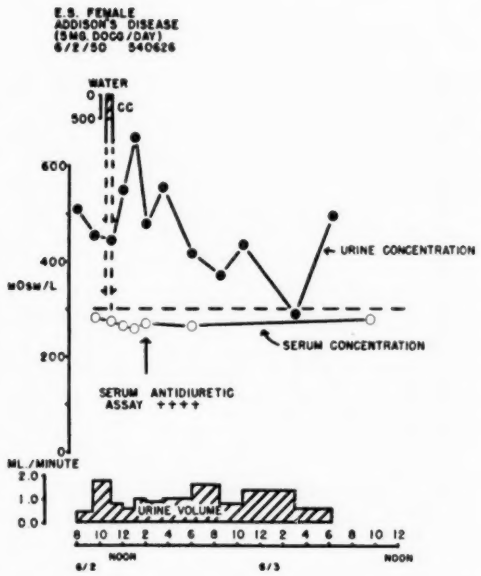

FIG. 1. THE ABNORMAL ANTIDIURETIC PATTERN IN A PATIENT WITH INADEQUATELY TREATED ADDISON'S DISEASE

Urine and serum total solute concentrations are plotted on the ordinate with time on the abscissa. Note that the serum total solute concentration is well below the normal level for man, indicated by the broken line. In spite of this the urine is concentrated and there is failure of prompt dilution of the urine following water administration. Antidiuretic activity was demonstrated in the serum at a time following water ingestion when the normal would show no antidiuretic activity in the serum.

does, in fact, reflect the low concentrations of serum sodium commonly associated with this condition. In spite of this the urine is found to be hypertonic to the serum. This finding is clearly not in accord with the normal physiology previously described (1).

It was first thought that this patient's osmotic regulating mechanism might be set to preserve some level of concentration lower than the normal—275 mOsm/L, say, instead of the normal 314 mOsm/L. If this were the case the patient should promptly excrete a water load with preservation of serum total solute concentration as does the normal (1). As seen in Figure 1, the expected dilution of the serum occurred following water administration but there was no diuresis or prompt dilution of the urine.

Furthermore, two hours following the water ingestion when the normal subject would have a polyuria with a dilute urine and would show no antidiuretic activity in his serum, this patient's urine was concentrated and her serum gave a strongly positive assay for antidiuretic activity. Though nothing can be said from this experiment regarding the source of the antidiuretic activity, it is apparent that this patient's osmotic regulating mechanism was not showing the normal response to a dilution of the serum. As a diuresis of dilute urine would obviously abolish this hypotonicity of the extracellular fluid, it is clear that this abnormal antidiuresis is responsible for the hypotonic state of the extracellular fluid.

That the ingested water load was sufficient to produce a normal diuretic pattern is shown in Figure 2. At this time the patient was receiving 100 mgs. of cortisone acetate intramuscularly daily. Her serum total solute concentration had returned to normal and the same dose of water resulted on two occasions in a prompt diuresis of dilute urine.

B. In congestive heart failure

Very low concentrations of sodium in the serum are seen in occasional patients with congestive heart failure who have been vigorously treated by measures to remove sodium from the body in the attempt to reduce edema. Unfortunately, all too commonly sodium is removed but extracellular fluid volume is not reduced proportionately and serum sodium concentrations fall.

Table II and Figure 3 show data from two such cases. Again we encounter low serum solute concentrations together with low concentrations of serum sodium. The urine, in spite of this, is found to be concentrated and there is no diuresis or dilution of the urine following water ingestion although the serum showed slight further dilution.

Assay of the serum of patient N.A.B. indicated the presence of antidiuretic activity at a time following water ingestion when the serum of a normal subject would have been free of antidiuretic activity. Thus the pattern of water excretion in this group of patients is found not to differ from

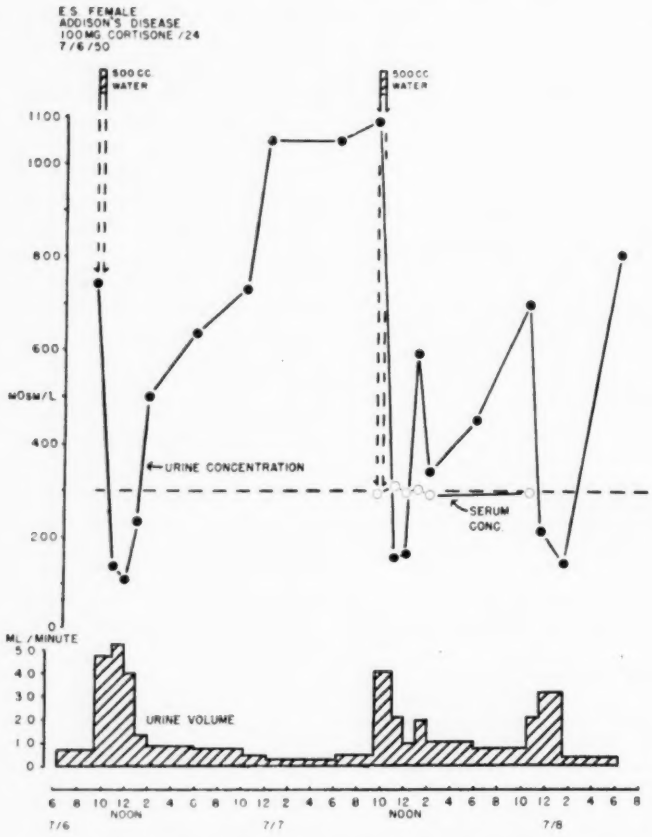

FIG. 2. THE NORMAL DIURETIC PATTERN ON TWO OCCASIONS IN THE SAME PATIENT WITH ADDISON'S DISEASE FROM WHOM THE DATA FOR FIGURE 1 WERE OBTAINED

In this instance the patient had been receiving 25 mgs. of cortisone acetate intramuscularly four times daily for seven days. Note that now serum total solute concentration is normal and the same dose of water that previously failed to cause a diuresis now is effective in causing prompt diuresis and dilution of the urine. Aside from a six-hour period following the two test water loads the patient was on an *ad lib* water intake. Thus the third episode of polyuria with dilution of the urine resulted from fluids spontaneously ingested.

that of the patient with uncontrolled Addison's disease. That adrenal insufficiency is not a factor in this group of patients is indicated by the very low concentrations of sodium in the urine shown in Table II and the low circulating eosinophile counts in this group—10 and 16 per cubic millimeter of fasting venous blood in subject N.A.B. on the day of study.

C. In dogs depleted of extracellular fluid electrolyte

It seemed that the factor common to both the above clinical groups might be a depletion of extracellular fluid electrolyte. If this were correct one should perhaps be able to convert a normal animal from the normal diuretic pattern to this abnormal pattern by depleting it of extracellular fluid electrolyte. Table III records the protocol

TABLE II

(3/26/51) N. A. B. Coronary heart disease and congestive failure

Hour	Serum		Urine		Na (*mEq/L*)	Assay
	Na (*mEq/L*)	Total solutes (*mOsm/L*)	Flow (*ml./min.*)	Total solutes (*mOsm/L*)		
0				576	2.0	
0–1			600 ml. water orally			
1	117	282	0.6	585		
2			0.8	576		
3	118	272	0.6	576	3.0	+67 (p = 0.03)
4			0.6	600		
5			0.8	570		
6	119	288	0.5	591		
8			0.6	576		
9			0.9	570		
10			0.4	594		
12			0.5	585		
14			0.4	591	5.0	
19			specimen lost			
21			1.0	585		
22			1.0	655		
25	119	279				

of a depletion study on Dog B presented in Figure 4. Parts A and C are the control hydration observations before and after the depletion (Part B) and are in every respect the normal response to a water load (1).

TABLE III

Part A. Hydration control, 12/4/50, Dog B (10.4 kilo)

Hour	Serum		Urine		Na (*mEq/L*)	Assay
	Na (*mEq/L*)	Total solutes (*mOsm/L*)	Flow (*ml./min.*)	Total solutes (*mOsm/L*)		
0	147	338		1562	74	
0–0.1			300 ml. water by stomach tube			
1			1.0	150		
2	143	318	2.2	82	6.5	+58 (p = 0.43)
3			0.5	375		
4	147	330	0.3	655		
5			0.1	1413	78	

Part B. Dog B depleted of 130 mEq Na by peritoneal dialysis, 12/8/50 (9.9 kilo)

0	122	295		600	1.9	
0–0.1			300 ml. water by stomach tube			
1			0.2	595		
2	121	280	0.3	585	0.8	+184 (p = 0.03)
3			0.3	680		
4			0.2	620		
5	119	273	0.4	600	1.3	

Part C. Hydration control after return to diet and salt, 12/11/50, Dog B (9.8 kilo)

0	147	327		1617	2.0	
0–0.1			300 ml. water by stomach tube			
1			0.2	640		
2	140	300	2.2	72	1.4	+22 (p = 0.43)
3			0.7	150		
4			0.1	720		
5	142	307	0.1	994	7.0	

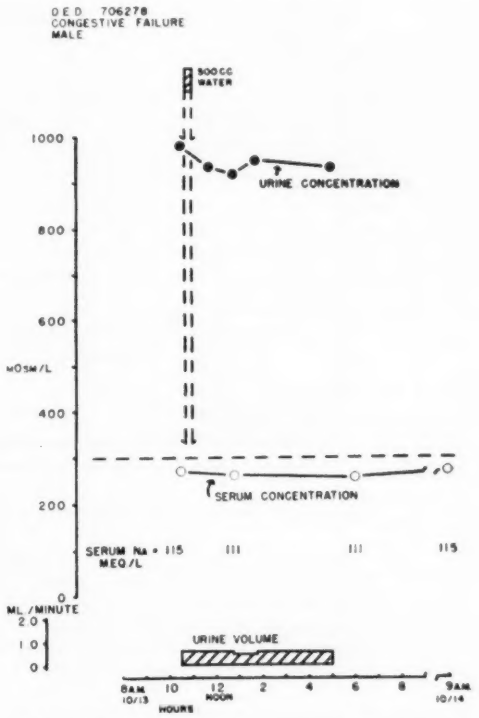

FIG. 3. THE ABNORMAL ANTIDIURETIC PATTERN IN A PATIENT WITH CONGESTIVE HEART FAILURE WHO DEVELOPED A VERY LOW CONCENTRATION OF SERUM SODIUM DURING TREATMENT OF HIS EDEMA

Note the dilute serum (271, 262, 256, and 272 mOsm/L, respectively) but the concentrated urine with complete absence of diuresis or dilution of the urine following water ingestion. The urine concentration remains not much below the maximum concentration for man. The urine sodium concentrations were 1.6, 1.8, 1.2, 1.5, and 1.5 mEq/L, respectively. These minimal urine sodium concentrations, in spite of the high urine total solute concentrations, indicate that the subject was conserving sodium as well as water.

Following the peritoneal dialysis with removal of nearly half the estimated normal sodium content of the extracellular fluid, this animal became listless, anorexic and oliguric showing loss of skin turgor and severe dehydration as described by Darrow and Yannet in their classic studies (5). The serum sodium was found to be low in association with a low total solute concentration. However, the urine was concentrated and there was

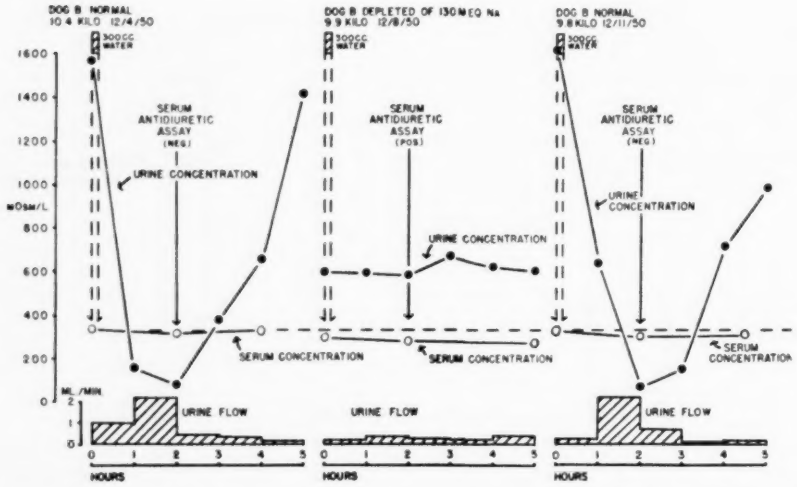

FIG. 4. THE EFFECT OF DEPLETION OF EXTRACELLULAR ELECTROLYTE ON THE DIURETIC PATTERN IN THE DOG

The right and left hand portions show the diuretic pattern before and following depletion. These are entirely normal diuretic patterns. The middle section shows conversion to the abnormal antidiuretic pattern following removal of 130 mEq of sodium by peritoneal dialysis. In this section we see again a dilute serum but a hypertonic urine and failure of dilution of the urine or diuresis following water administration. Antidiuretic activity was demonstrated in the serum during depletion but not in the controls.

failure of diuresis and dilution of the urine following the same water load which prior to and following depletion resulted in a satisfactory diuresis. Also antidiuretic activity was demonstrable in the serum of the depleted animal whereas no significant evidence of antidiuretic activity could be obtained in the two control periods. One animal, whose state of depletion was maintained by a sodium-free intake for eight days, excreted a persistently concentrated urine and exhibited this abnormal antidiuresis when tested on the second, fifth, and eighth days. Thus the normal dog depleted of extracellular fluid electrolyte mimics the abnormal antidiuretic mechanism described above in patients with inadequately treated Addison's disease and certain cases of congestive heart failure that develop low concentrations of serum sodium.

D. In dogs during administration of small amounts of posterior pituitary extract

The finding of antidiuretic activity in the serum of patients and dogs exhibiting this abnormal antidiuretic phenomenon obviously suggested an overactivity of the supraopticohypophyseal antidiuretic mechanism in spite of the dilution of extracellular fluid which normally inhibits its activity. If such were the case one should be able again to reduplicate this abnormal pattern by the continuous administration of posterior pituitary antidiuretic hormone.

Figure 5 shows the results on water excretion on the third day of administration of 2.0 units of Pitressin Tannate in Oil (Lot K888G) twice daily intramuscularly to Dog C together with 1 liter of water daily by stomach tube. A control hydration study is shown in the same figure. A similar study in Dog B is shown in Figure 6 in which half the dose of Pitressin was used. Again we see the abnormal antidiuretic pattern repeated in all details studied.

The same results have been obtained in humans on five occasions using the smaller dose of Pitressin Tannate in Oil. Undoubtedly similar results could be obtained with considerably smaller amounts of Pitressin Tannate in Oil but the difficulty in measuring smaller volumes of this prepa-

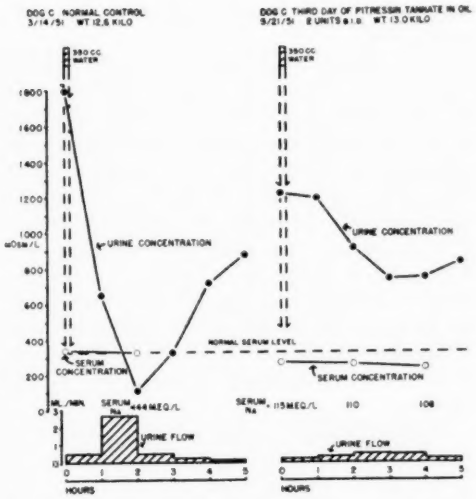

FIG. 5. THE EFFECT OF POSTERIOR PITUITARY EXTRACT ON WATER DIURESIS IN THE DOG

On the left is shown a normal control diuretic pattern. On the right is shown the abnormal antidiuretic pattern produced by 2.0 units of Pitressin Tannate in Oil administered twice daily for two days together with 1,000 ml. of water by stomach tube daily. Again we see a dilute serum but a concentrated urine and failure of diuresis following water administration. This animal gained little weight during Pitressin administration and in appearance simulated the signs of salt depletion shown at the time of peritoneal dialysis (see text). It is interesting that under these circumstances, in spite of large exogenous antidiuretic hormone administration, the urine concentration was not nearly so great as that achieved by spontaneous endogenous antidiuretic hormone activity during water restriction.

ration prevented further reductions in dosage. The dosage used in man approximates the estimated physiological endogenous output of the posterior pituitary (6, 7).

As can be seen in the figures the gain in body weight observed with Pitressin administration was insufficient to explain the dilution on the basis solely of water retention. A large diuresis of sodium and chloride probably occurred during the period of Pitressin administration such as may occur in man (8). This possibility is supported by the ensuing loss of weight to over 1 kilo below the control weight following the post-Pitressin diuresis when the serum sodium and total solute concentrations had returned to normal.

DISCUSSION

The finding of low concentrations of serum sodium in patients who were elaborating distinctly hypertonic urines was not sufficient evidence for concluding that the normal regulation of diuresis and antidiuresis was not operating. Retention of other substances in the extracellular fluid could theoretically have compensated for low sodium and chloride concentrations. When the total solute concentration of the serum, as well as its sodium and chloride concentrations, was found to be low this theoretical possibility was excluded. It was at once evident that the normal physiology did not pertain in these cases.

Baldes and Smirk (9) emphasized that it was the acute drop in serum total solute concentration following water ingestion which acts as the stimulus for water diuresis rather than the absolute concentration. They found that the gradual decrease in serum total solute concentration produced in normal subjects by low salt diets plus sweating did not give rise to a state of diuresis.

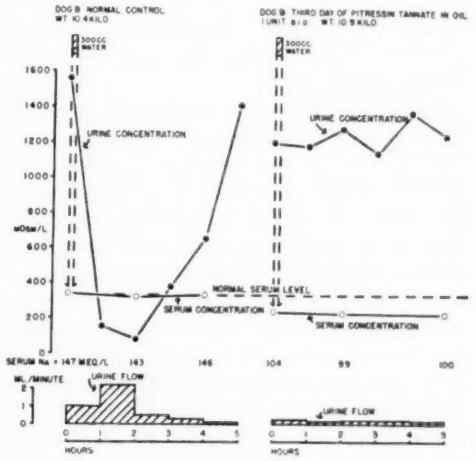

FIG. 6. THE EFFECT OF POSTERIOR PITUITARY EXTRACT ON WATER DIURESIS IN THE DOG

On the left is shown a normal control diuretic pattern. On the right is shown the abnormal antidiuretic pattern produced by 1.0 unit of Pitressin Tannate in Oil administered twice daily for two days with 1,000 ml. of water by stomach tube daily. The serum was found to be very dilute but the urine concentrated following Pitressin administration and water ingestion produced no diuretic response.

Furthermore, a dose of water given to such a depleted subject was noted to produce a prompt diuresis. Thus their subjects still responded, as does the normal, to protect the existing level of serum solute concentration. However, the drop in serum total solute concentration they produced was only some 9 and 13 mOsm/L in two instances.

Our data clearly indicate that in some cases exhibiting depression of serum total solute concentration of 40 to 50 mOsm/L below the normal a diuresis or dilution of urine may not occur in response to water administration. Serum solute concentration in these patients is thus not promptly protected from further dilution. In cardiac patients showing more moderate reductions of serum sodium and solute concentration (serum sodium concentration circa 130–135 mEq/L and total solute concentrations of 290–300 mOsm/L) an intermediate diuretic response was frequently observed. Such patients had a delayed diuresis with hypotonic urine occurring only five or more hours following the water ingestion. This intermediate response was also seen in patients with Addison's disease who were receiving inadequate therapy with cortisone or desoxycorticosterone and in our dogs when the depletion by intraperitoneal dialysis was less severe.

Obviously a delay in the rate of water absorption from the gastrointestinal tract could account for absence of diuresis and dilution of the urine. The observed dilution of serum total solute concentration is proof of absorption of the ingested water. Early in this study 3 liters of water were given as the test water load to a patient with untreated Addison's disease. The severe water intoxication with coma that ensued was convincing evidence that absorption of water from the gut did in fact occur. With administration of the major portion of the water load intravenously to one case of Addison's disease and one of the cardiac subjects, the abnormal antidiuretic pattern was not altered.

Because of the preeminence of sodium in determining serum total solute concentration, excretion of urine which is hypertonic but low in sodium concentration could correct the extracellular fluid hypotonicity. The concentrated urine of the edematous subjects and salt depleted dogs was in fact very low in sodium concentration but was of insufficient volume to restore serum tonicity to the normal level.

It was difficult to distinguish in the normal but depleted dog and in the untreated patient with Addison's disease what effects were solely the result of altered renal hemodynamics secondary to insufficiency of extracellular fluid volume. Thus the profound dehydration in the dogs, though not associated with shock, may be expected to reduce renal blood flow and glomerular filtration rate. How much of the failure of diuresis and the persistence of hypertonic urines can be attributed to the decreased glomerular filtration rate which must of necessity accompany depletion of extracellular fluid volume is obviously of importance to this problem.

A direct answer to this question is not currently available. However, the patients with congestive failure demonstrating this abnormal antidiuretic pattern still had excessive extracellular fluid volumes as evidenced by the clinical presence of edema. They frequently had normal concentrations of blood urea nitrogen suggesting that glomerular filtration rates were not markedly reduced. Furthermore, in one 31 year old female with severe rheumatic heart disease and intractable congestive failure the inulin clearance was found to be 106 ml./min./1.73 m^2 (10) or some 88% of normal mean. This clearance value was obtained two hours following the ingestion of 1,000 ml. of water when the serum sodium concentration was 120 mEq/L, the rate of urine flow less than 1.0 ml./min. and the urine concentrated (956, 910, and 904 mOsm/L for the three 30-minute periods, respectively). On the other hand, one patient with posterior and anterior pituitary insufficiency, to be reported (8), who was never observed spontaneously to produce a hypertonic urine even with severe dehydration (with serum sodium concentrations as high as 173 mEq/L), was found to have an inulin clearance of 63 ml./min./1.73 m^2 (10) or some 53% of normal mean. This clearance was obtained with no preceding hydration at a time when her serum sodium was 151 mEq/L, her urine flow 6.2, 5.7, and 6.2 ml./min. with urine concentrations of 141, 148, and 148 mOsm/L for the three clearance periods, respectively. These observations of a nearly normal glomerular filtration rate in one patient exhibiting the abnormal antidiuretic pattern and a distinctly reduced fil-

tration rate in another patient, who characteristically had a very dilute urine, make it seem improbable at the present time that reduction in glomerular filtration rate is alone responsible for the abnormal antidiuretic pattern described. Somewhat in support of this is the recent report by Holmes and Cizek (11) that some of their dogs, depleted of extracellular fluid electrolyte by osmotic diuresis with sucrose, failed to have a prompt water diuresis following water administration, but showed no reduction in glomerular filtration rate. The osmotic diuretic used by these workers, however, admittedly may produce renal damage. Clearly, more data will be necessary to settle this crucial point.

This abnormal pattern of antidiuresis, characterized by a dilute extracellular fluid but a concentrated urine with failure of diuresis or dilution of the urine following water administration, suggested abnormal activity of the neurohypophyseal antidiuretic mechanism. This pattern was reproduced readily by injections of posterior pituitary extract into normal dogs and man. It is the antithesis of what one sees in posterior pituitary insufficiency during water deprivation (1), namely, a concentrated extracellular fluid but a dilute urine. In the present situation body water is unduly conserved while with posterior pituitary insufficiency it is wasted. The demonstration of antidiuretic activity in the serum perhaps also supports this view although nothing can be said regarding the specificity of the assay for antidiuretic material of posterior pituitary origin. At the present time we are attempting to repeat the salt depletion experiment on a post-hypophysectomized dog to test whether loss of neurohypophyseal function will abolish the abnormal pattern described.

In 1939, Winter, Ingram and Gross (12) showed that adrenalectomized cats had a drop in serum sodium and chloride concentrations while diabetes insipidus cats when adrenalectomized did not develop a reduction in these concentrations. Treatment of the latter group with sufficient Pitressin to control the polyuria caused a return of low serum sodium and chloride concentrations. Their work seems to show conclusively that the abnormal antidiuretic pattern, at least in the subject with adrenal insufficiency, is dependent upon an intact posterior pituitary gland. As the pattern is so similar in other conditions associated with a dilute extracellular fluid (excluding the anuric subject over-loaded with water), it is strongly suspected that over-activity of the posterior pituitary antidiuretic mechanism is common to them all.

It is common clinical practice to consider a low serum sodium concentration as evidence of sodium depletion. As indicated in the introduction to this paper, however, present physiological concept that posterior pituitary activity is regulated by changes in tonicity of extracellular fluid makes the very occurrence of a low serum sodium concentration difficult to explain. As sodium is the major osmotically active constituent of the extracellular fluid its loss could be masked, so far as concentration change is concerned, by rejection of a proportional amount of water by the kidney. In view of the large volume of glomerular filtrate, even in the sodium depleted subject, the occurrence of dilution of extracellular fluid sodium can only mean that something has influenced the renal tubules to reabsorb not 145 mEq of sodium with each liter of water, as normally from the glomerular filtrate, but rather 120 or so mEq per liter of water reabsorbed. Is this influence, which causes a relatively greater than normal reabsorption of water than of sodium by the renal tubules of the sodium depleted subject, antidiuretic hormone?

The water content of the body is the balance between the intake of water and its precursors in the diet, on the one hand, and the continuous sensible and insensible loss of water from the body on the other. A cessation or a reduction of water intake below the minimal obligatory volume of urine and insensible loss of water would soon correct a dilution of the extracellular fluid. The desire for water, which caused some of these patients with dilute extracellular fluids to complain bitterly when attempts were made clinically to correct their state of dilution by water restriction, attested to the fact that the "thirst center," as well as the kidney, was cooperating to maintain the hypotonic state of the extracellular fluid. This observation suggests that there are other stimuli for thirst than an increase in tonicity of the extracellular or intracellular fluid (13).

Our findings are in accord with those of Darrow and Yannet in dogs (5) and McCance and Widdowson in man (14), who noted that experimental salt deficiency in both species produced an impaired water diuresis. We do not find ex-

planation in our more acute salt depletion studies for the polyuria and polydipsia reported by Cizek and his colleagues (15) for chronic salt depletion studies in dogs. Our results are in general confirmatory of those of Martin, Herrlich and Fazekas (16) who reported finding antidiuretic activity in the urine of adrenalectomized cats and salt depleted cats. In the latter group the urinary antidiuretic activity was said to be inversely proportional to the serum sodium level.

The question arises as to whether one type of stimulus could be sufficient to activate supraopticohypophyseal function and liberation of antidiuretic hormone both in the normal and the salt depleted animal. Verney's work is convincing that tonicity change of the serum constitutes the effective stimulus in the normal animal. The results presented here seem to exclude tonicity change as a possible stimulus to antidiuretic activity in the salt depleted animal, and some function of change in extracellular fluid volume has been considered the effective stimulus under these circumstances.[4] Thus "volume-receptors" are postulated much as Verney has postulated "osmoreceptors." Physiologically these two receptors might conceivably have been identical. In the normal animal loss of water without salt from the extracellular fluid would result in a decrease in volume of that compartment as well as an increase in its concentration. In the salt depleted animal loss of salt and of water would likewise reduce extracellular fluid volume. A decrease in volume then might be the effective stimulus in both instances. However, the clear-cut experiments of Verney in which he infused hypertonic solutions into the internal carotid arteries of hydrated dogs causing prompt antidiuresis definitely excludes such a unitary stimulus. It is most difficult to see how a further intravascular infusion in a hydrated animal can in any way be considered to cause a reduction of any part of the extracellular fluid compartment. Thus it seems that at the present we are left with two distinct but effective stimuli for prolonged supraopticohypophyseal activity,[5] tonicity change and possibly volume change of some portion of the extracellular fluid compartment.[6]

Normally the total effective solute concentration of the extracellular fluid is jealously guarded. It is apparent, however, that a mechanism exists which effects conservation of water irrespective of serum solute levels. Teleologically, it seems that with depletion of extracellular fluid electrolyte and the resulting threat of death from vascular insufficiency, the tonicity of extracellular fluids is sacrificed and water is retained in an attempt to ex-

[4] There has been recent work showing that in the adrenalectomized animal the rate at which the liver destroys the antidiuretic activity of posterior pituitary extracts is considerably slower than in the normal (17, 18). This finding is cited as the explanation for the inability of the adrenalectomized animal to have a water diuresis (19). It would seem that one encounters logical difficulties in attempting to explain the entire defect in water excretion on this basis. Regardless how slow inactivation of antidiuretic hormone might be, the dilution of extracellular fluid in the subject with Addison's disease should prevent further release of antidiuretic hormone. When all circulating antidiuretic hormone finally would be inactivated a water diuresis should then occur and continue until serum total solute concentration returns to normal. This is not what happens as serum total solute concentration persists at low levels. Hence it seems that there is required an over-production of antidiuretic hormone as well as the demonstrated delayed inactivation to explain the entire disturbance in water excretion. Admittedly a delayed inactivation of antidiuretic hormone in combination with a setting of the "osmoreceptors" to preserve some level of total solute concentration lower than normal could also explain the results observed in the patients with Addison's disease. Until more data on this point are available which will permit a physiological basis for subdividing the conditions associated with hypotonicity, the authors include them in one group.

[5] Other stimuli have been shown effectively to cause release of antidiuretic hormone and inhibition of water diuresis. Emotion (20, 21), pain (22, 3), exercise (23, 20), quiet standing (24, 25) and syncope (26) all inhibit water diuresis presumably by causing release of antidiuretic hormone. The short duration of the antidiuresis produced by these stimuli place them into quite a different category than the protracted antidiuresis we are discussing. The antidiuresis of quiet standing and syncope, however, may operate through the same volume-receptor mechanism postulated here (or presso-receptor of Brun, Knudsen and Raaschou [24]).

[6] It is quite possible that the sequence of events leading from extracellular fluid volume insufficiency to the presumed increased activity of the neurohypophysis is not nearly as direct as here indicated. The volume insufficiency may impair circulation in certain organs so as to alter metabolism causing local formation of substances that exhibit antidiuretic activity through release of posterior pituitary hormone. The demonstration by Shorr and his associates (27) of the antidiuretic action of the hepatic vasodepressor ferritin (VDM) constitutes an example of one such possibility.

pand volume. This disparity between salt and water retention which produces a hypotonic serum can probably only arise under the artificial conditions of a low salt intake but an *ad lib* water intake. If salt is made available, the expansion or reexpansion of extracellular fluid volume will be accomplished with preservation of isotonicity. The artificial conditions have been utilized simply to separate out this different mechanism for water retention which is thus shown to operate irrespective of extracellular fluid solute concentration.

That the edematous cardiac subject apparently utilizes the same mechanism grossly to over-expand extracellular fluid volume that the normal uses to correct a deficit, adds perhaps further interest to this phenomenon. It indicates clearly that it is not change in total extracellular fluid volume, but more likely insufficiency of some critical portion, probably intravascular,[7] which sets off this volume-expanding antidiuretic mechanism. Something about the failing myocardium produces perhaps a volume insufficiency in this critical zone which is met by a retention of water and of salt (if the latter is available in the intake) with a generalized expansion of extracellular fluid volume. Such a generalized expansion of extracellular fluid volume in a salt depleted but otherwise normal animal might sufficiently replenish vascular volume so as to correct the insufficiency in this critical zone. With a severely damaged myocardium the retention of fluid and electrolyte may not accomplish this end and the stimulus for further expansion would then persist with massive edema formation as the end result. The low serum oncotic pressures associated with edema of nephrotic syndrome and cirrhosis may in some way act to set off this same trigger mechanism.

In congestive heart failure venous pressures are increased while in the animal depleted of extracellular fluid electrolyte with resultant reduced vascular volume, venous pressures seem to be reduced. If each is utilizing the same pathways for expanding extracellular fluid volume, then it appears unlikely that a high venous pressure is in itself the effective stimulus for edema formation and attention should perhaps be concentrated on looking for local insufficiencies on the arterial side.

Borst has stated (29) that acute hemorrhage (which by definition is loss of isotonic fluid) results in a concentrated urine low in chloride concentration. This would seem to be another instance of a volume conserving antidiuretic mechanism.

As shown in the tables of results the sodium depleted animal and the edematous cardiac subjects were maximally conserving sodium as indicated by its very low concentrations in the urines. Expansion of extracellular fluid volume would occur with sodium as well as water retention if the former were available in the intake. Thus the response is to expand volume by retention of its two major constituents, salt and water. This study has dealt with only one phase of the process of water retention, but it seems that in edema formation both are occurring and the salt retention is the more important of the two, as the usual edematous subject with essentially normal concentrations of serum sodium can handle large water loads with little, if any, difficulty (30). Does the same "volume" stimulus which apparently activates posterior pituitary activity similarly influence adrenal cortical activity with resultant salt retention (31)? The low circulating eosinophile counts in our cardiac subjects is perhaps evidence for this.

SUMMARY AND CONCLUSIONS

It is shown that patients with inadequately treated Addison's disease, certain cases of congestive heart failure who develop low serum sodium concentrations during treatment of their edema, and normal dogs depleted of extracellular fluid electrolyte show an abnormality of water excretion. This consists of a dilution of the serum but, in spite of this, a concentrated urine and a failure to have a prompt water diuresis or dilution of the urine following administration of water. This antidiuretic pattern is reduplicated by continuous action of small amounts of posterior pituitary extract in normal dogs and man. It is considered at present that this abnormal antidiuretic pattern does represent a continued activity of the supraopticohypophyseal antidiuretic system in the

[7] From studies of the effect of plasma volume increase in normal subjects produced by infusions of albumin solutions Welt and Orloff (28) have recently hypothecated that uncomplicated expansion of the plasma volume initiates a diuresis of water by supressing the activity of the posterior pituitary gland.

presence of a dilute extracellular fluid which normally would inhibit its activity. It is suggested that the stimulus for posterior pituitary activity in the subject depleted of extracellular fluid electrolyte is an insufficiency of some function of volume in a crucial portion of the extracellular fluid volume, possibly intravascular. The significance of finding this phenomenon in the edematous subject is discussed.

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REFERENCES

1. Leaf, A., and Mamby, A. R., The normal antidiuretic mechanism in man and dog: its regulation by extracellular fluid tonicity. J. Clin. Invest., 1952, **31**, 54.
2. Verney, E. B., Absorption and excretion of water: antidiuretic hormone. Lancet, 1946, **2**, 781.
3. Verney, E. B., Antidiuretic hormone and the factors which determine its release. Proc. Roy. Soc. London, Series B, 1947, **135**, 25.
4. Berry, J. W., Chappell, D. G., and Barnes, R. B., Improved method of flame photometry. Indust. & Engin. Chem., Analyt. Ed., 1946, **18**, 19.
5. Darrow, D. C., and Yannet, H., The changes in the distribution of body water accompanying increase and decrease in extracellular electrolyte. J. Clin. Invest., 1935, **14**, 266.
6. Shannon, J. A., The control of the renal excretion of water. II. The rate of liberation of the posterior pituitary antidiuretic hormone in the dog. J. Exper. Med., 1942, **76**, 387.
7. Lauson, H. D., Eder, H. A., Chinard, F. P., Cotzias, G. C., and Greif, R. L., Estimation of the rate of antidiuretic hormone secretion in normal man. Federation Proc., 1948, **7**, 69.
8. Leaf, A., and Mamby, A. R., Some hormonal aspects of water excretion in man. To be published.
9. Baldes, E. J., and Smirk, F. H., The effect of water drinking, mineral starvation and salt administration on the total osmotic pressure of the blood in man, chiefly in relation to the problems of water absorption and water diuresis. J. Physiol., 1934, **82**, 62.
10. Miller, B. F., Leaf, A., Mamby, A. R., and Miller, Z. B., The validity of the endogenous creatine clearance as a measure of glomerular filtration rate in the diseased human kidney. Unpublished data.
11. Holmes, J. H., and Cizek, L. J., Observations on sodium chloride depletion in the dog. Am. J. Physiol., 1951, **164**, 407.
12. Winter, C. A., Ingram, W. R., and Gross, E. G., Effect of Pitressin injections upon the serum electrolytes and water exchange of cats with diabetes insipidus and adrenal insufficiency. Am. J. Physiol., 1939, **127**, 64.
13. Dill, D. B., Life, Heat and Altitude. Physiological Effects of Hot Climates and Great Heights. Harvard University Press, Cambridge, Mass., 1938.
14. McCance, R. A., and Widdowson, E. M., The secretion of urine in man during experimental salt deficiency. J. Physiol., 1937, **91**, 222.
15. Cizek, L. J., Semple, R. E., Huang, K. C., and Gregersen, M. I., Effect of extracellular electrolyte depletion on water intake in dogs. Am. J. Physiol., 1951, **164**, 415.
16. Martin, S. J., Herrlich, H. C., and Fazekas, J. F., Relation between electrolyte imbalance and excretion of an antidiuretic substance in adrenalectomized cats. Am. J. Physiol., 1939, **127**, 51.
17. Eversole, W. J., Birnie, J. H., and Gaunt, R., Inactivation of posterior pituitary antidiuretic hormone by the liver. Endocrinology, 1949, **45**, 378.
18. Birnie, J. H., Inactivation of posterior pituitary antidiuretic hormone by liver extracts. Federation Proc., 1950, **9**, 12.
19. Gaunt, R., and Birnie, J. H., Hormones and Body Water. Charles C Thomas, Springfield, Ill., 1951.
20. Rydin, H., and Verney, E. B., The inhibition of water-diuresis by emotional stress and by muscular exercise. Quart. J. Exper. Physiol., 1938, **27**, 343.
21. O'Connor, W. J., The control of urine secretion in mammals by the pars nervosa of the pituitary. Physiol. Rev., 1947, **22**, 30.
22. Verney, E. B., Absorption and excretion of water: antidiuretic hormone. Lancet, 1946, **2**, 739.
23. Pickford, M., Control of the secretion of antidiuretic hormone from the pars nervosa of the pituitary gland. Physiol. Rev., 1945, **25**, 573.
24. Brun, C., Knudsen, E. O. E., and Raaschou, F., The influence of posture on the kidney function. I. The fall of the diuresis in the erect posture. Acta med. Scandinav., 1945, **122**, 315.
25. Brun, C., Knudsen, E. O. E., and Raaschou, F., The influence of posture on the kidney function. II. Glomerular dynamics in the passive erect posture. Acta med. Scandinav., 1945, **122**, 332.
26. Brun, C., Knudsen, E. O. E., and Raaschou, F., On the cause of post-syncopal oliguria. Acta med. Scandinav., 1945, **122**, 486.
27. Shorr, E., Baez, S., Zweifach, B. W., Payne, M. A., Mazur, A., and Metz, D. B., The antidiuretic action of the hepatic vasodepressor ferritin (VDM) and its occurrence in conditions associated with antidiuresis in man. Tr. A. Am. Physicians, 1950, **63**, 39.

28. Welt, L. G., and Orloff, J., The effects of an increase in plasma volume on the metabolism and excretion of water and electrolytes by normal subjects. J. Clin. Invest., 1951, **30**, 751.

29. Borst, J. G. G., The maintenance of an adequate cardiac output by the regulation of the urinary excretion of water and sodium chloride: an essential factor in the genesis of oedema. Acta med. Scandinav., 1948, Suppl., **207**.

30. Schemm, F. R., A high fluid intake in the management of edema, especially cardiac edema. I. The details and basis of the regime. Ann. Int. Med., 1942, **17**, 952.

31. Leaf, A., and Couter, W. I., Evidence that renal sodium excretion by normal human subjects is regulated by adrenal cortical activity. J. Clin. Invest., 1949, **28**, 1067.

PULMONARY ARTERY DIASTOLIC PRESSURE: ITS RELATIONSHIP TO PULMONARY ARTERIOLAR RESISTANCE AND PULMONARY "CAPILLARY" PRESSURE [1]

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In the systemic circulation, one ordinarily may assume increased peripheral resistance when the diastolic pressure is elevated (1). It is the purpose of this paper to ascertain whether a similar relationship exists within the lesser circulation of man.

MATERIAL

Subjects were selected at random from the medical wards of the Cincinnati General Hospital. Some were normal convalescents; others were suffering from various types of heart and lung disease (Table I). Satisfactory data were obtained in 54 patients.

METHOD

Subjects were studied in the fasting condition, sedated by 0.1 or 0.2 gm. of seconal. Catheterization of the right side of the heart was done as described previously (2). Pulmonary "capillary" pressure was obtained by the method of Hellems and co-workers (3). The criteria of satisfactory pulmonary "capillary" pressures were *1*) the nature of the pressure curve, showing "a" and "c" waves; *2*) the peripheral location of the catheter; and *3*) the securing of blood saturated with oxygen from the wedged catheter tip. In some instances no blood could be obtained from the wedged catheter. In the majority of instances a double lumen catheter was used, so that pulmonary artery and pulmonary "capillary" pressures could be recorded simultaneously. In some instances, a single lumen catheter was employed; in these cases pulmonary "capillary" pressure was recorded first; then the catheter tip was quickly withdrawn into the proximal portion of the pulmonary artery so that almost simultaneous recording of the pulmonary artery pressure could be made. Recordings of pressures were made with the Hathaway blood pressure recording apparatus.

Cardiac outputs were determined by the direct Fick method as outlined before (2). Mixed venous samples were obtained from the proximal portion of the pulmonary artery. Duplicate samples of expired air were analyzed for CO_2 and O_2 and required to check within 0.03%. Duplicate blood samples, obtained during the collection of expired air, were analyzed for O_2 on the Van Slyke manometric apparatus and required to check within 0.2 vol. %.

Resting cardiac outputs were obtained after the catheter had been in place 15 to 30 minutes or more. Immediately after taking blood and gas samples for the output, pressure recordings were made. Mean pulmonary artery and pulmonary "capillary" pressures were determined by planimetry. Measurements were made over two or more respiratory cycles. A point 10 cm. above the table on which the subject lay was taken as the zero point in pressure measurements.

Pulmonary arteriolar resistance was calculated from the formula

$$R = \frac{PA - PC}{CO} \times 1{,}332,$$

where R = arteriolar resistance in dynes sec./cm.$^{-5}$,
PA = mean pulmonary artery pressure, mm. Hg,
PC = mean pulmonary "capillary" pressure, mm. Hg,
CO = cardiac output in cc./sec.

RESULTS

These are shown in Table I and Figures 1 through 7. In Figure 1 is shown a scatter diagram plotted logarithmically indicating the observed relationship between pulmonary artery diastolic pressure and pulmonary arteriolar resistance. The correlation (4) between the two was high ($r = 0.92$; $p = < 0.001$). In Figures 2 and 3 are logarithmic graphs indicating the relationships between pulmonary arteriolar resistance and pulmonary artery systolic and mean pressures. It is noteworthy that even closer correlation was found here. The correlation coefficient of resistance and systolic pressure was 0.95 ($p = < 0.001$); the

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TABLE I

	Pulmonary "capillary" mean pressure, *mm. Hg*	Pulmonary artery pressure, *mm. Hg*		Cardiac output, *L./min.*		Pulmonary arteriolar resistance, *dynes sec./cm.*$^{-5}$	Arterial O_2 sat., *% of capacity*
		Mean	Systolic/diastolic	Index	Total		
1. B. M. (F, 57) Bronchiectasis	9	29	38/17	2.7	4.0	397	84.1
2. I. W. (F, 29) Post-partum myocardosis	18	25	37/15	3.3	4.9	114	94.2
3. M. C. (M, 73) Normal	10	13	22/6	2.3	3.5	78	85.9
4. S. J. (M, ?68) HCVD, no failure	14	25	36/20	2.3	4.5	202	94.7
5. C. C. (M, 46) Normal (conv. empyema)	12	17	25/11	2.8	5.0	74	90.3
6. J. S. (M, 75) Hypertension	22	26	39/22	2.6	4.3	89	93.5
7. J. B. (M, 63) Carcinoma of lung	15	26	36/21	2.7	4.2	202	94.4
8. O. B. (M, 37) Conv. pneumonia	7	15	26/9	3.2	5.5	106	89.4
9. J. H. (M, 62) Hypertension	6	14	19/9	2.5	5.2	110	86.5
10. G. A. (M, 59) Cor pulmonale, diaphragmatic hernia	18	45	61/35	2.9	4.9	452	75.5
11. N. J. (M, 22) Conv. rheum. fever	10	14	22/8	3.3	5.5	57	90.9
12. N. M. (F, 42) Rheumatoid arthritis	7	17	24/11	2.6	4.1	209	91
13. A. H. (F, 56) HCVD	8	17	30/11	4.8	6.9	103	94.1
14. C. P. (M, 30) Conv. pneumonia	11	18	25/13	2.5	4.7	116	95.1
15. M. S. (M, 49) Mitral stenosis	23	54	70/30	2.0	3.7	666	86.5
16. M. B. (M, 60) HCVD, Silicosis	7	18	27/14	2.3	4.2	213	96.1
17. P. S. (M, 43) Malnutrition	4	10	18/7	3.7	5.9	85	99.3
18. J. R. (M, 36) Conv. pneumonia	4	16	23/9	3.0	5.5	162	89.8
19. W. B. (M, 37) Conv. pleurisy	7	13	21/9	4.5	8.0	62	89.6
20. C. M. (M, 49) Rheumatoid arthritis	7	18	26/9	3.0	5.1	170	90.3
21. J. S. (M, 39) Hemoptysis ? cause	7	19	32/10	2.6	4.8	189	83
22. L. J. (M, 20) Fibrosis lung	14	27	36/21	3.0	7.3	133	92.7
23. L. A. (M, 43) Neuritis, malnutrition	9	14	24/9	4.3	7.9	47	93.1
24. V. H. (M, 27) Epilepsy, lung abscess	6	10	15/6	3.2	5.1	69	95.6
25. E. B. (F, 28) Beri-beri	22	32	36/25	6.1	9.9	87	86.6
26. H. H. (M, 39) Bulbar paresis	9	15	16/7	3.0	4.9	83	95.6
27. F. H. (F, 38) Nutritional cirrhosis	7	16	23/10	5.4	7.6	93	92.3
28. G. Y. (M, 46) Conv. pneumonia	11	17	24/13	2.3	4.3	113	94.1
29. C. F. (M, 50) Conv. pneumonia	15	24	37/18	1.9	3.2	217	96.6
30. H. P. (M, 48) CNS lues	6	16	23/11	3.0	5.4	142	94.1
31. C. H. (F, 49) Paresis	8	20	23/10	3.4	5.8	160	96.3
32. S. M. (M, 40) Conv. pneumonia	7	14	21/9	3.1	5.2	101	96.5

TABLE 1 (*Continued*)

	Pulmonary "capillary" mean pressure, *mm. Hg*	Pulmonary artery pressure, *mm. Hg*		Cardiac output, *L/min.*		Pulmonary arteriolar resistance, *dynes sec./cm.*$^{-5}$	Arterial O_2 sat., *% of capacity*
		Mean	Systolic/diastolic	Index	Total		
33. E. J. (M, 43) Normal heart, chronic leukemia	7	16	24/12	3.3	6.5	113	93.7
34. M. L. (F, 52) Emphysema	6	21	34/16	2.4	3.6	337	89.3
35. F. B. (F, 20) Sickle cell anemia	13	19	25/10	5.6	8.6	56	83.0
36. R. J. (M, 69) Pneumoconiosis	7	17	30/10	2.1	3.6	223	89.1
37. F. J. (M, 46) HCVD, LMCA, thrombosis	11	23	34/12	2.1	4.2	214	97.4
38. O. M. (M, 39) HCVD, failure	17	35	50/29	1.5	2.4	593	94.9
39. A. B. (M, 46) Comp. HCVD, lues, optic atrophy	12	22	36/14	3.9	7.8	102	95
40. G. A. (M, 60) Cor pulmonale	19	74	119/51	1.8	3.3	1331	55.2
41. I. R. (M, 31) Mitral stenosis	19	29	45/18	2.3	3.9	211	90.8
42. C. G. (M, 66) Normal heart, frontal lobe atrophy	6	14	23/7	3.0	5.5	126	91.9
43. W. E. (M, 56) Carcinoma lung, multiple myotonia	16	35	52/28	3.7	5.9	256	73.7
44. J. M. (M, ?47) Pneumonia, carcinoma lung	8	12	22/7	3.6	5.7	53	90.8
45. E. M. (M, 37) Paresis	10	18	24/9	3.6	6.1	98	90.4
46. G. Y. (M, 48) Normal	6	17	24/8	4.7	8.9	104	92.2
47. F. T. (M, 52) Metastatic carcinoma lung	6	25	36/14	3.3	5.6	270	91.6
48. J. S. (M, 52) Emphysema	9	20	28/15	2.1	3.7	223	92.5
49. J. B. (M, 53) Emphysema, cor pulmonale	12	34	55/20	3.2	5.5	321	78.1
50. R. M. (M, 50) Paresis, luetic A.I.	8	14	25/10	2.3	4.2	110	100
51. H. F. (M, 43) Silicosis, ?pulmonary fibrosis	11	29	53/19	2.2	4.2	351	94
52. H. W. (M, 60) LMCA, thrombosis	5	12	20/8	2.2	3.3	150	95.9
53. H. S. (M, 51) Emphysema	6	32	64/17	3.2	4.8	415	85.6
54. C. D. (M, 26) Eisenmenger's tetralogy	9	102	140/80	2.2	4.2	1790	84.8

correlation coefficient for resistance and mean pressure was 0.94 ($p = < 0.001$).

When pulmonary "capillary" pressure was plotted against pulmonary arteriolar resistance on a logarithmic graph (Figure 4), no significant correlation was found ($r = 0.23$; $p = > 0.05$). In Figure 5, pulmonary arteriolar resistance is plotted against arterial oxygen saturation on semilogarithmic paper. A significant negative correlation was found ($r = -0.54$; $p = < 0.001$). Correlation between pulmonary artery diastolic pressure and pulmonary "capillary" pressure was determined and is shown on semilogarithmic paper (Figure 6). A significant correlation was observed ($r = 0.45$; $p = < 0.001$). No significant correlation between arterial oxygen saturation and cardiac output was observed (Figure 7; $r = 0.007$; $p = > 0.9$).

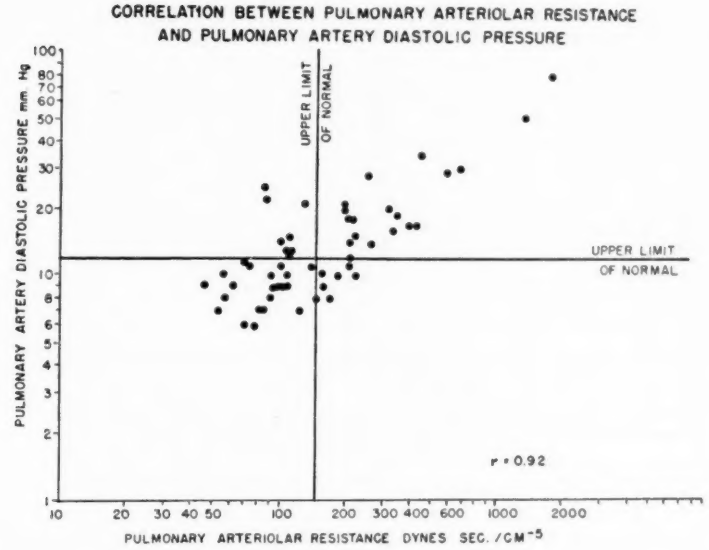

FIG. 1

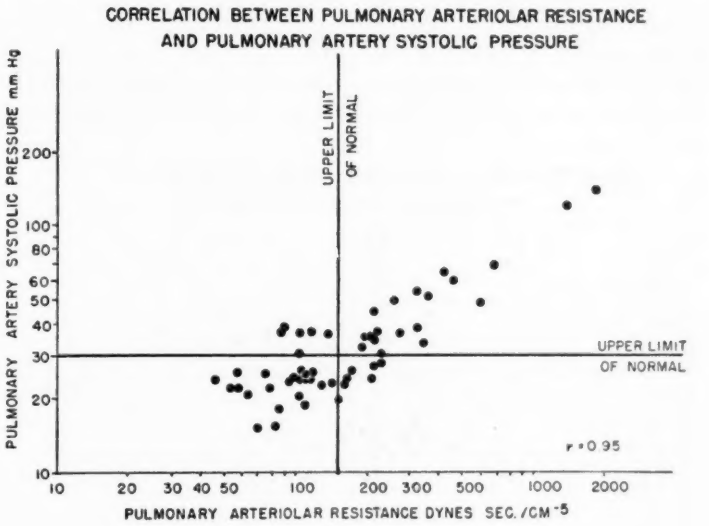

FIG. 2

DISCUSSION

It has been suggested that the elevation of pulmonary artery diastolic pressure commonly seen in emphysema is a reflection of the increased pulmonary vascular resistance found in that condition (5). Although this may well be true, it would appear from our data that it would be dangerous to predict arteriolar resistance from the pulmonary artery diastolic pressure. Although a high degree of correlation is shown between pulmonary

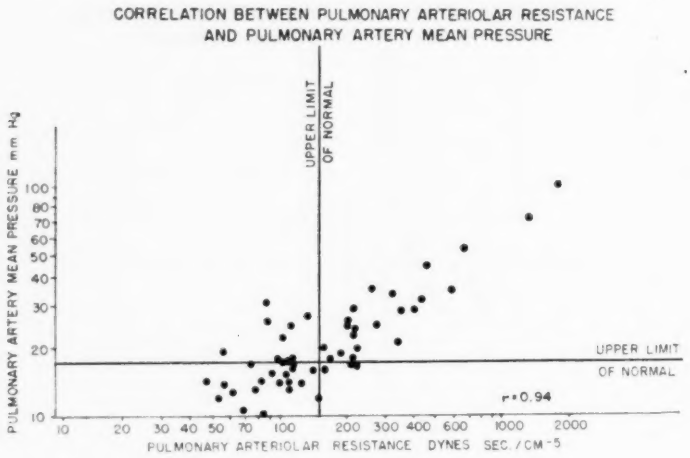
CORRELATION BETWEEN PULMONARY ARTERIOLAR RESISTANCE
AND PULMONARY ARTERY MEAN PRESSURE
PULMONARY ARTERY MEAN PRESSURE mm Hg
UPPER LIMIT OF NORMAL
UPPER LIMIT OF NORMAL
r=0.94
PULMONARY ARTERIOLAR RESISTANCE DYNES SEC./CM-5

FIG. 3

artery diastolic pressure and pulmonary arteriolar resistance, there is considerable scatter; furthermore, eight patients had normal resistance with high diastolic pressure and seven patients had high resistance with normal diastolic pressure. In addition, an even higher degree of correlation with pulmonary arteriolar resistance was found with regard to pulmonary artery mean pressure and pulmonary artery systolic pressure than with regard to the pulmonary diastolic pressure. It must be borne in mind that the pulmonary artery pressure depends upon cardiac output and left atrial pressure as well as pulmonary vascular resistance. One would interpret the above findings to indicate that a rise in pulmonary arteriolar resistance increases the pulmonary artery pressure, and that

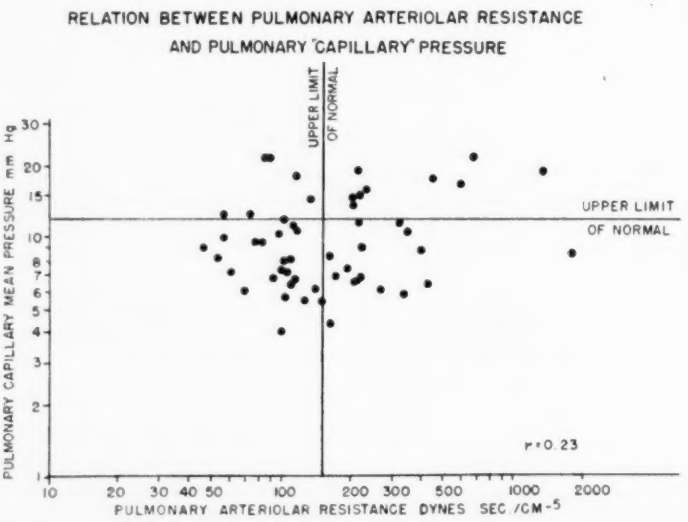
RELATION BETWEEN PULMONARY ARTERIOLAR RESISTANCE
AND PULMONARY "CAPILLARY" PRESSURE
PULMONARY CAPILLARY MEAN PRESSURE mm Hg
UPPER LIMIT OF NORMAL
UPPER LIMIT OF NORMAL
r=0.23
PULMONARY ARTERIOLAR RESISTANCE DYNES SEC./CM-5

FIG. 4

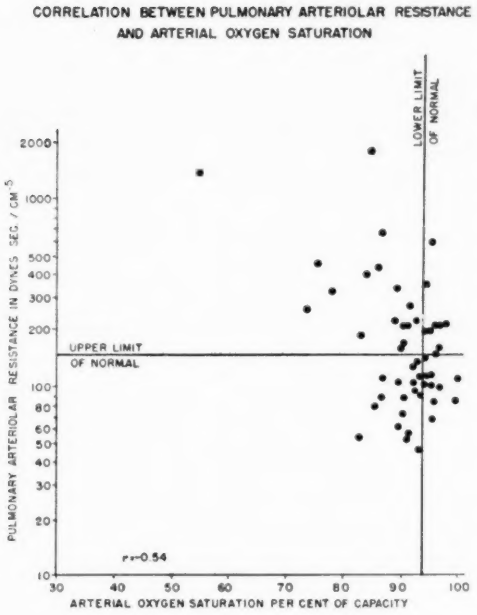
CORRELATION BETWEEN PULMONARY ARTERIOLAR RESISTANCE AND ARTERIAL OXYGEN SATURATION
LOWER LIMIT OF NORMAL
UPPER LIMIT OF NORMAL
r=-0.54
PULMONARY ARTERIOLAR RESISTANCE IN DYNES SEC. / CM.-5
ARTERIAL OXYGEN SATURATION PER CENT OF CAPACITY

FIG. 5

the increase is as great or greater in the mean and systolic pressures than in the diastolic.

In view of the statement that a rise in left atrial pressure would be expected to produce an increase in pulmonary artery diastolic pressure (6), the significant correlation observed between this pressure and pulmonary "capillary" pressure is of interest since the latter is considered to vary with left atrial pressure. There was a fairly wide range of scatter here, however. It is also important to note that in only seven of the 54 subjects did pulmonary "capillary" pressure exceed the pulmonary artery diastolic pressure. Even in these instances the differences were small and could be explained by the error of the experimental method.

The failure to find correlation between the height of pulmonary "capillary" pressure and the amount of pulmonary arteriolar resistance does not necessarily conflict with the observations of Dexter (7), since our data contain no pulmonary "capillary" pressures above 25 mm. Hg at which level protective constriction of the pulmonary arteries is presumed to occur in order to prevent pulmonary oedema.

The finding of a significant negative correlation between arterial oxygen saturation and pulmonary arteriolar resistance is of interest, in view of the evidence that hypoxia produces pulmonary artery vaso-constriction (6, 8). We believe, however, that these data alone would not necessarily permit one to draw that conclusion. Correlation and causation are not the same. It is likely that conditions such as pulmonary emphysema which are commonly associated with arterial hypoxia produce pulmonary arterial hypertension through a mechanism of which hypoxia is but a part.

Our failure to find correlation between cardiac output and arterial oxygen saturation is in agreement with the observations of Borden and coworkers (5) who found no evidence of such correlation in 24 cases of emphysema, but is in disagreement with some theories as to the cause of high cardiac output in cor pulmonale (6). One should not conclude, however, that our results necessarily indicate no relation between cardiac output and hypoxia in emphysema, since our data were ob-

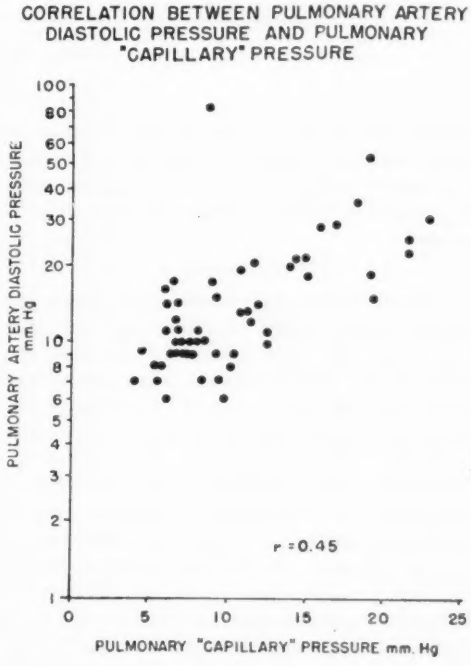
CORRELATION BETWEEN PULMONARY ARTERY DIASTOLIC PRESSURE AND PULMONARY "CAPILLARY" PRESSURE
r=0.45
PULMONARY ARTERY DIASTOLIC PRESSURE mm. Hg
PULMONARY "CAPILLARY" PRESSURE mm. Hg

FIG. 6

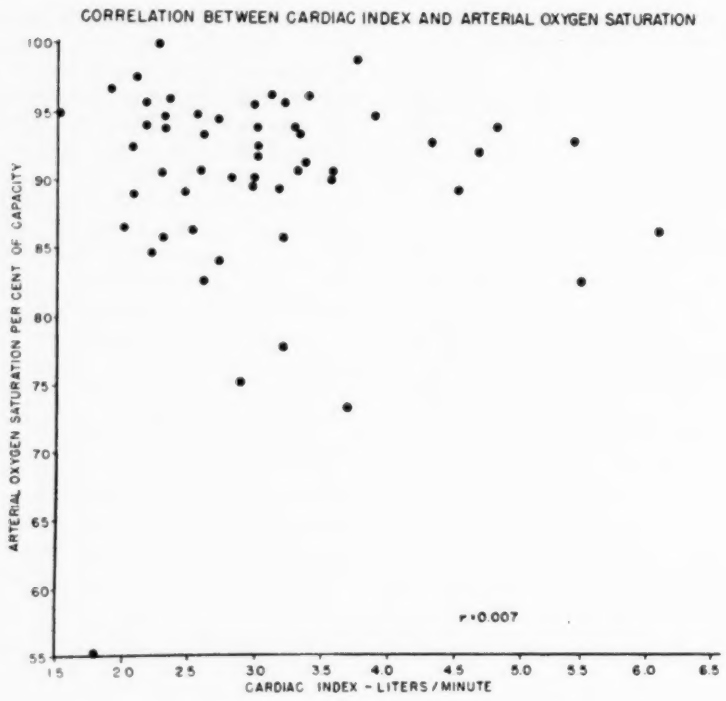

FIG. 7

tained from patients suffering from a variety of heart diseases. Unlike the heart in emphysema, the hearts in some of our subjects would be unable to respond to a stress situation by an increase in output.

SUMMARY

A study of pulmonary arteriolar resistance, pulmonary artery systolic, diastolic, and mean pressures, and pulmonary "capillary" pressure was made in 54 human subjects, some normal and some suffering from a variety of diseases. Significant correlation was found between pulmonary arteriolar resistance and each of the following: pulmonary artery systolic, mean, and diastolic pressures. The correlation of resistance with diastolic pressure was no closer than with mean or systolic pressure. A significant negative correlation between pulmonary arteriolar resistance and arterial oxygen saturation was found. Significant correlation between pulmonary "capillary" pressure and pulmonary artery diastolic pressure was observed. No significant correlation was found between arterial oxygen unsaturation and cardiac output. It is concluded that an increase in pulmonary arteriolar resistance produces an elevation in pulmonary artery mean, systolic, and diastolic pressures. There is no evidence that increased resistance produces a disproportionate elevation in pulmonary diastolic pressure as compared to mean and systolic pressures.

REFERENCES

1. Best, C. H., and Taylor, N. B., The Physiological Basis of Medical Practice: A Text in Applied Physiology. Williams & Wilkins Co., Baltimore, 1950, Ed. 5.
2. Fowler, N. O., Westcott, R. N., Scott, R. C., and McGuire, J., The effect of nor-epinephrine upon pulmonary arteriolar resistance in man. J. Clin. Invest., 1951, **30**, 517.
3. Hellems, H. K., Haynes, F. W., Dexter, L., and Kinney, T. D., Pulmonary "capillary" pressure in animals estimated by venous and arterial catheterization. Am. J. Physiol., 1948, **155**, 98.

4. Fisher, R. A., Statistical Methods for Research Workers. Oliver & Boyd, London, 1950, Ed. 11.
5. Borden, C. W., Wilson, R. H., Ebert, R. V., and Wells, H. S., Pulmonary hypertension in chronic pulmonary emphysema. Am. J. Med., 1950, **8**, 701.
6. Cournand, A., Some aspects of the pulmonary circulation in normal man and in chronic cardiopulmonary diseases. Circulation, 1950, **2**, 641.
7. Dexter, L., Pulmonary circulatory dynamics in health and disease, at rest. Bull. N. E. Med. Center, 1949, **11**, 240.
8. Westcott, R. N., Fowler, N. O., Scott, R. C., Hauenstein, V. D., and McGuire, J., Anoxia and human pulmonary vascular resistance. J. Clin. Invest., 1951, **30**, 957.

PRODUCTION OF INCREASED RENAL SODIUM EXCRETION BY THE HYPOTONIC EXPANSION OF EXTRACELLULAR FLUID VOLUME IN RECUMBENT SUBJECTS [1]

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The renal excretion of sodium is obviously determined by the difference between the amount filtered through the glomeruli and the amount reabsorbed by the tubules. In the isolated, perfused kidney, increasing the filtered sodium chloride either by raising filtration rate or salt concentration in the perfusate increases the excretion of salt, but even in this preparation changes in tubular reabsorption play a significant role (1, 2). While there is no reason to doubt that alteration in the filtered load may act in the same direction in the intact animal as in the isolated kidney, changes in tubular reabsorption appear to be of considerable importance in regulating sodium excretion (3), as possibly first suggested by Starling and Verney (1) slightly over a quarter century ago: "Our experiments show that even a concentration of sodium chloride in the blood far greater than normal is unable *per se* to bring about its elimination. It seems, therefore, that some hormone or hormones arising in the tissues pass by way of the blood stream to the kidney, making it cognizant of the state of the tissues in their content of water and chloride and moderating the reabsorptive mechanism in accordance with the needs of the organism at the time." The evidence that adrenal cortical secretion plays a role in controlling tubular sodium reabsorption need not be recounted here, nor the fact that the anterior pituitary gland exercises a certain degree of control over the adrenal cortex. However, even in the absence of the adrenal cortex, sodium balance can be achieved if the intake is sufficiently large, while in the absence of the anterior pituitary, serious disturbances in sodium metabolism rarely occur unless the individual is subjected to stresses beyond the ability of the autonomous regulatory powers of the kidney and possibly of the adrenal cortex to compensate.

Under certain circumstances the rate of sodium excretion can be correlated with the concentration of sodium in the extracellular fluid. Such is not invariably the case. Thus the augmented sodium excretion associated with an increased dietary intake of salt may be related to a rise in serum sodium, but under these circumstances there is a concomitant expansion of extracellular fluid volume. On the other hand in primary water depletion, when serum sodium concentration also rises, but extracellular volume contracts, there is actually diminished urinary excretion of sodium (4), suggesting that the volume of extracellular fluid (or some associated factor) rather than its osmolar concentration is the stimulus to salt retention and excretion (5). Accordingly it seemed of interest to determine whether it was the volume of water in the body as a whole or in some compartment that played a role in controlling sodium excretion.

Welt and Orloff (6) have infused into semirecumbent subjects 50 to 100 gm. of salt-poor human albumin in 25 per cent solution, thereby increasing calculated plasma volume by from 22 to 51 per cent in nine experiments. Neither during the period of infusion (one hour for 50 gm., two hours for 100 gm.) nor during the ensuing two hours was there any increase in the rate of renal excretion of Na or Cl. Creatinine and urea clearances and the concentrations of sodium and chloride in the serum were unchanged.

They also infused 1,875 ml. of 4 per cent albumin in 5 per cent glucose into one normal subject, expanding calculated plasma volume by 31 per cent and causing a small fall in serum sodium

[1] Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

and chloride concentration. Creatinine clearance, and sodium and chloride excretion were unaltered during the two hours of infusion and in the following two hours, although a striking water diuresis occurred.

In other experiments these same workers were unable to demonstrate that the infusion of 2,280 ml. of 4, 5 or 6 per cent albumin in normal saline produced any changes in sodium or chloride excretion other than those which occurred during the control infusion of 2,280 ml. of normal saline without albumin. Similarly, in subjects whose extracellular volume had been expanded by the infusion of 2,500 ml. of normal saline, the subsequent infusion of 200 ml. of 25 per cent albumin produced no changes in sodium excretion other than those which occurred with a control infusion of 200 ml. of normal saline.

Since Welt's and Orloff's subjects receiving iso- or hypo-oncotic albumin solutions were moderately hydropenic by virtue of abstinence from fluids for 10 to 14 hours before, as well as during the experiments, we have repeated the studies with iso-oncotic albumin in 5 per cent glucose, employing subjects who were undergoing "maximum" water diuresis at the time (see Methods). Under these conditions there occurred a slight transient increase in sodium excretion of the same magnitude as that which followed the administration of an amount of sodium acetyltryptophanate equal to that in the albumin. Since these subjects, whose antidiuretic hormone production presumably had already been suppressed by the water load, exhibited no further increase in diuresis, these observations substantiate the hypothesis of Welt and Orloff that the water diuresis which they observed is the result of inhibition by the expanded plasma volume of the secretion of antidiuretic hormone.

It thus appears that the expansion of plasma volume by the infusion of hypo-, iso-, or hyper-oncotic solutions of albumin in normal recumbent subjects is without significant effect on the renal excretion of sodium and chloride.

The effect of increasing the total volume of water in the body on the urinary excretion of sodium is not as clearly defined. Crutchfield and Wood (7) found that in recumbent subjects whose urine volumes were relatively low during a control hour the ingestion of 1 liter of tap water led to an increased excretion of sodium whereas those whose control urine volumes were high excreted less sodium after water loading. Seldin and Tarail (8) noted only a slight initial increase in sodium excretion in two normal subjects who accumulated positive water balances of approximately 2.5 liters by drinking water at a rate of 20 to 22 ml./min. Blomhert (9) made similar observations in subjects ingesting 2 liters of water over a period of one hour. We have observed slight transient increases in sodium excretion under similar circumstances (10). That these changes are not merely due to a "washing-out" effect involving the renal dead-space and delay time is indicated by the fact that the excretion of creatinine and other solutes undergoes no similar changes. In an endeavor further to elucidate this matter we have administered 10 units of posterior pituitary extract (Pitressin, Parke, Davis & Co.) to two recumbent normal subjects after a control period of two hours, and then, two hours after the Pitressin injection, while its antidiuretic effect was still present, had the subjects ingest 3 liters of tap water. Sodium excretion averaged 198 micro-eq./min. before Pitressin, 122 micro-eq./min. during the first two hours after Pitressin and 160 micro-eq./min. during the next two hours of hydration. Serum sodium averaged 139.3 meq./l. before hydration and 131.6 meq./l. after. Plasma volume, estimated from changes in hemoglobin, hematocrit and total serum protein, increased by 3.5 per cent, indicating that a significant quantity of the water had actually been absorbed from the gastro-intestinal tract. It would thus appear that expansion of total body water has a relatively minor effect on the excretion of sodium, at least when serum sodium concentration is lowered.

In an endeavor to ascertain the effect of expansion of the extracellular compartment, experiments were devised in which the *volume* of extracellular water was *expanded* at the same time that the *concentrations* of sodium and chloride therein were *reduced*. Under these conditions any renal or extra-renal effects ascribable to an increased concentration of these ions either in extracellular fluid or in glomerular filtrate could be eliminated from consideration.

METHODS

The subjects were essentially healthy male adults admitted for the study of minor complaints and free from significant physical abnormalities. They came to the

laboratory one to two hours after a breakfast low in salt, were weighed, arterialized blood was withdrawn (11) without stasis from an antecubital vein, and 1,500 ml. of tap water was imbibed during a period of one-half to one hour. They stood up to void every half hour, following which they drank an amount of tap water slightly in excess of the urine volume so as to replace insensible loss and maintain the body weight 1.5 Kg. greater than the initial weight.[2] After a satisfactory diuresis had been in progress for two hours blood for analysis was withdrawn and the intravenous infusion of 2 liters [3] of a solution containing 112 meq. of sodium, 90 meq. of chloride and 22 meq. of bicarbonate per liter was commenced at a rate of approximately 20 ml./min. The ingestion of water every half hour was continued so that at the conclusion of the infusion the body weight was maintained 3.5 Kg. above the initial weight. Blood was withdrawn at the conclusion of the infusion and two hours later. The study was terminated three or four hours after completion of the infusion. Although ideally it would have been preferable to have employed the same subjects for both the sitting and recumbency studies, this was not done because of the rather arduous nature of these experiments for the subjects.

Analytic methods and formulae for calculation of changes in plasma volume were identical to those described in a recent report from this laboratory (12).

RESULTS

The rates of urine flow and electrolyte excretion, sodium concentration and creatinine clearance, together with analyses made on venous blood and calculated changes in plasma volume are given in Table I. The rate of sodium excretion is shown graphically in Figure 1.

The absence of any significant change in calculated plasma volume together with the fall in concentration of electrolytes in extracellular water following the establishment and during the maintenance of a tap water load of 1,500 ml. is consistent with the belief that, as absorbed, this water is distributed throughout the entire volume of body water. Calculation of the extracellular volume from the chloride balance and chloride concentration in extracellular water before and after the infusion indicated increases of 12 to 16 per cent following the infusion of 2,000 ml. of hypotonic sodium chloride and bicarbonate solution (containing the amount of sodium normally present in 1,600 ml. of extracellular fluid). Thus the calculated expansion of extracellular volume, also reflected in the calculated increases in plasma volume, agree with the anticipated changes.

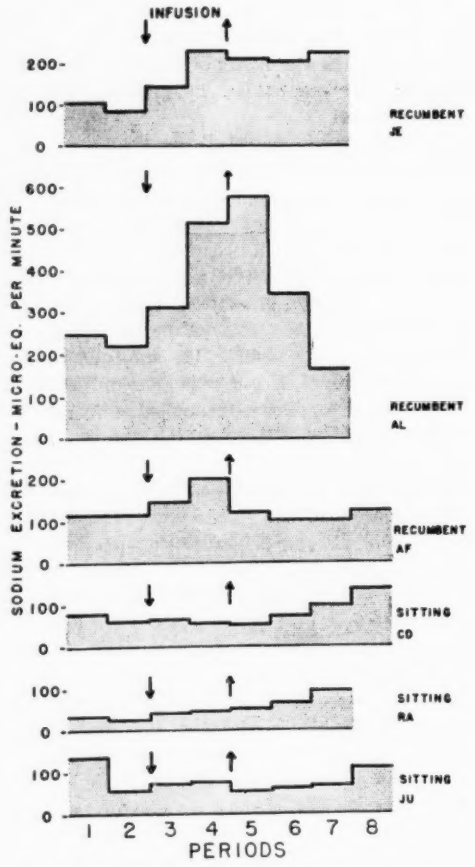

FIG. 1. THE EFFECT OF THE INTRAVENOUS INFUSION OF HYPOTONIC SODIUM CHLORIDE AND BICARBONATE SOLUTION ON SODIUM EXCRETION IN WATER-LOADED SUBJECTS

Note that subject A. L., whose weight was 110 Kg., received 3 liters; the others 2 liters.

[2] Without such preliminary and sustained water loading it is difficult to prevent a rise in serum electrolyte concentration following the infusion of hypotonic solutions since an initial effect of expanded extracellular volume appears to be that of inhibition of antidiuretic hormone secretion, leading to the excretion of urine which is more dilute than the infused solution and thus obligatorily raising the concentration of electrolytes in the serum (9, 12). Furthermore, such loading leads to continued large urine volumes, minimizing collection and timing errors as well as renal dead-space changes.

[3] In the case of Subject A. L. whose weight was 110 Kg., 3 liters were infused.

TABLE 1

The effect of the intravenous infusion of hypotonic sodium chloride + bicarbonate solution on urinary excretion of water and electrolytes, creatinine clearance and on the constituents of the blood and the volume of the plasma

Subject Weight Height Position	Period*	Urine						Serum†						Blood†		$PV_2/PV_1 \times 100$†‡	
		Flow	Na	K	Cl	C_{cr}	Na conc.	Na	K	Cl	CO_2	Cr	TP	Hgb.	Hct.	From Hct. and Hgb.	From TP
		ml./min.	*micro-eq./min.*	*micro-eq./min.*	*micro-eq./min.*	*ml./min.*	*meq./l.*	*meq./l.*	*meq./l.*	*meq./l.*	*meq./l.*	*mg./100 ml.*	*gm.%*	*gm.%*	*vol.%*	*per cent*	*per cent*
J. E.	1	14.9	104	194	170	138	7.0	133.4	4.6	102.1	26.5	0.91	6.4	14.5	48.1	100.0	100.0
69.69 Kg.	2	11.9	87	116	98	139	7.3										
167 cm.	3§	12.2	145	60	102	145	11.9	129.1	4.4	99.0	26.2	0.79	6.8	14.7	48.8	97.2	94.1
Recumbent	4§	14.6	228	68	132	150	15.6										
	5	13.5	208	54	117	142	15.4	127.9	4.2	97.2	26.6	0.77	5.9	13.7	46.4	109.3	108.5
	6	12.9	202	48	119	140	15.7										
	7	13.3	223	40	122	143	16.8	125.6	4.2	97.4	25.8	0.79	5.8	13.6	46.1	110.7	110.0
A. L.	1	17.9	249	150	289	139	13.9	139.6	4.3	105.3	27.8	1.05	7.3	14.0	45.2	100.0	100.0
110.58 Kg.	2	19.1	220	114	252	134	11.5										
188 cm.	3§	20.9	307	112	333	131	14.7	134.2	4.2	102.3	27.0	1.00	7.1	13.9	45.9	99.4	102.8
Recumbent	4§	24.3	518	111	507	141	21.3										
	5	24.4	580	94	548	141	23.8	134.6	3.7	101.3	26.1	0.90	6.2	12.5	42.1	118.3	117.8
	6	18.9	348	74	337	137	18.4										
	7	14.3	167	74	169	132	11.7	133.6	3.9	104.5	26.2	0.92	6.3	13.0	41.8	114.4	115.8
A. F.	1	11.9	117	117	157	111	9.8	137.2	4.6	105.2	29.2	0.81	7.1	14.7	47.0	100.0	100.0
63.92 Kg.	2	11.7	117	88	127	116	10.0										
178 cm.	3§	10.7	147	53	129	115	13.7	133.7	4.4	102.0	26.9	0.74	6.9	14.6	48.0	98.8	102.9
Recumbent	4§	11.3	203	50	187	125	18.0										
	5	9.1	121	44	115	118	13.3	130.7	4.0	100.7	27.0	0.67	6.0	13.2	44.8	116.0	118.3
	6	8.4	102	40	84	118	12.2										
	7	8.6	102	36	69	117	11.9	130.5	4.1	100.8	27.3	0.69	5.9	12.7	42.9	124.7	120.3
	8	9.6	126	34	77	125	13.1										
C. D.	1	9.3	80	117	148	114	8.6	138.8	5.0	106.0	29.3	1.12	6.5	15.4	47.2	100.0	100.0
76.37 Kg.	2	8.6	61	77	101	109	7.1										
175 cm.	3§	7.6	66	70	93	110	8.7	136.0	4.4	102.0	27.5	1.11	6.2	14.8	47.0	104.4	104.8
Sitting	4§	6.7	57	74	84	109	8.5										
	5	7.3	55	56	64	128	7.7	131.0	4.0	102.2	25.1	0.96	5.8	13.3	46.9	116.4	112.0
	6	8.9	71	43	62	130	8.0										
	7	10.8	98	30	69	127	9.1	134.5	3.9	102.3	25.4	0.98	6.0	13.8	43.4	119.6	108.3
	8	12.4	138	35	72	132	11.1										
R. A.	1	11.8	34	78	45	122	2.9	137.3	4.4	105.3	28.7	0.99	7.4	15.3	48.8	100.0	100.0
61.06 Kg.	2	10.1	27	52	37	122	2.7										
169 cm.	3§	9.3	42	52	41	128	4.5	128.3	4.4	102.3	26.0	0.88	7.1	15.5	49.1	98.1	104.1
Sitting	4§	9.9	47	76	46	126	4.7										
	5	9.5	55	63	42	128	5.8	128.2	4.2	101.3	25.9	0.87	5.9	13.6	45.0	120.8	125.4
	6	9.5	68	45	42	127	7.2										
	7	10.2	97	35	39	126	9.5	130.0	4.1	104.5	26.4	0.85	6.2	14.0	44.3	118.9	119.4
J. U.	1	12.3	136	75	175	118	11.1	139.7	4.2	106.7	27.4	0.91	7.1	15.1	46.9	100.0	100.0
63.33 Kg.	2	8.7	56	58	97	105	6.4										
170 cm.	3§	9.7	71	62	109	119	7.3	133.0	4.2	102.6	26.9	0.89	6.8	14.3	46.2	107.0	104.0
Sitting	4§	10.2	77	62	118	123	7.5										
	5	8.6	53	53	85	118	6.2	130.7	4.0	102.7	26.4	0.81	5.8	12.7	41.3	131.4	122.4
	6	8.4	60	44	72	118	7.1										
	7	8.6	68	37	71	116	7.9	131.4	4.0	100.8	26.2	0.85	6.1	13.3	41.2	125.7	116.4
	8	9.8	110	36	81	121	11.2										

* Periods were of 60 minutes duration.
† Initial serum and blood values were determined prior to water loading. Subsequent determinations were performed at the beginning of the indicated periods.
‡ $PV_2/PV_1 \times 100$ indicates the calculated ratio of the new to the original plasma volume expressed as per cent.
§ Period of intravenous infusion.

In each observation with the subject recumbent, sodium excretion rose during the infusion and remained elevated for several hours. This increased excretion of sodium was in every instance associated with a rise in its concentration in the urine as well as with an increased rate of urine flow. The dependence of urine flow upon electrolyte and particularly sodium excretion, during states when antidiuretic hormone activity may be presumed to be minimal or absent previously described by us (13), has been considered to conform with the view that the water available for facultative excretion is related to the distal load of sodium (14). It will be noted that in every instance the concentrations of sodium, chloride and bicarbonate in the extracellular water fell during the infusion of the hypotonic solution, while creatinine clearance remained unchanged. Thus the filtered load of sodium was actually decreased at the time during which the excretion rate was increasing.

The total sodium excretion during the first five hours after beginning the infusion averaged 13.4 per cent of the amount infused in the recumbent subjects and only 4.4 per cent in the seated subjects.

In contrast to the effects in recumbent subjects, little augmentation of sodium excretion above the pre-infusion rate occurred in the seated subjects, and that only several hours after the infusion was completed. However, this rise in excretion is to be contrasted with the decline which might have been anticipated without the infusion (10). Whether this late rise represents a simple temporal delay in the phenomena observed in the recumbent subjects is not apparent.

DISCUSSION

The augmented excretion of sodium uniformly observed with subjects in the recumbent position at a time when the serum electrolyte concentrations were reduced and creatinine clearances unchanged can only be explained by diminished tubular reabsorption of sodium. In view of the fact that the depression of serum electrolyte concentration was unaccompanied by any shift in ionic pattern one can hardly ascribe the increased sodium excretion to its obligation by chloride—an interpretation which might be placed on the increased sodium excretion which accompanies an infusion of isotonic saline (12), and which occurs in the sitting as well as recumbent positions.

It is conceivable that the lowering of the oncotic pressure of the plasma served as the stimulus for diminished reabsorption of sodium. However, a reduction of equal magnitude occurred in the experiments with the subjects sitting, in which sodium excretion was little or not at all augmented. A similar argument may be raised against the unlikely hypothesis that the decreased concentration of serum sodium, with the concomitant fall in electrolyte concentration within the tubule cells, serves as a stimulus for sodium rejection or that an increase in renal interstitial fluid as part of the general expansion of extracellular volume leads to diminished sodium reabsorption.

That the locus of action of expanded extracellular volume is in the cephalad portion of the body is suggested by the lesser increase in sodium excretion in the sitting position. The observation of Harrison and associates (15, 16), that in sitting subjects increasing intracranial pressure by means of an inflated blood pressure cuff about the neck produces an augmented sodium excretion, is consistent with this hypothesis. Evidence has already been presented (12) indicating that the secretion of antidiuretic hormone is responsive to volume changes within the cranium. It might well be that the anterior pituitary or some intracranial center which influences its activity is likewise sensitive to volume changes or some factor associated with them, and that the release of salt-retaining adrenal hormones is thus modified, as suggested by Peters and colleagues (17) who studied a salt-wasting syndrome associated with cerebral disease. An hypothesis suggested is that "the excitation of the anterior pituitary gland as it relates to the secretion of adrenocorticotrophic hormone may be under a modifying influence from some nucleus or tract in the central nervous system. A lesion at such a site might disorganize an automatic sequence that normally leads to the secretion of ACTH and in turn of salt retaining hormone from the adrenal cortex" (17). An alternate hypothesis is that the central nervous system can directly modify the tubular reabsorption of salt.

The apparent converse of the above syndrome has also been reported. Hypernatremia and hyperchloremia associated with little or no salt excretion in the urine have been reported by Allott (18), Sweet and his coworkers (19), MacCarty and Cooper (20), and Higgins and associates (21) in

patients with serious brain damage, all of whom eventually succumbed. Luetscher's and Blackman's (22) cases of hypernatremia in association with brain damage ascribed to sulfonamides, although similar to the above cases, were all complicated by concomitant renal damage. In all these cases of hypernatremia it might well be that the difficulty resided at least in part in the failure of the supra-optico-hypophyseal system to respond normally to hypertonicity, although clear-cut diabetes insipidus was not noted. Furthermore Lewy and Gassmann (23) have produced hyperchloremia and hypochloruria but not polyuria in cats by inducing lesions in the paraoptic nuclei.

Previous studies (6, 9, 12) have demonstrated that expansion of extracellular volume (plasma and interstitial fluid) appears to diminish tubular reabsorption of water via decreased antidiuretic (ADH) activity. The present observations suggest that expanded extracellular volume diminishes tubular reabsorption of sodium. If, as seems not unreasonable, the converse of these propositions holds, namely, that diminished extracellular volume (or some associated factor) in the cephalad portion of the body leads to *1*) sodium retention through increased tubular reabsorption and *2*) water retention in excess of sodium through increased ADH activity, one may be in a position to explain the occurrence of these phenomena in certain cases of decompensated hepatic cirrhosis with ascites. In this condition the obviously increased volume of fluid below the diaphragm is not infrequently associated with clinical evidence of dehydration of the head, neck, arms and upper chest. These patients characteristically retain sodium with avidity, but in addition frequently exhibit hyponatremia (24, 25) often accompanied by relatively scanty urine of high specific gravity suggestive of ADH activity which indeed has been demonstrated by Ralli and colleagues (26). With improvement in liver function, the serum sodium tends to rise and the hematocrit to fall, the latter suggesting that an expansion of plasma volume has occurred, since it is unlikely that clinical improvement would be accompanied by either diminished blood formation or increased blood destruction. Although the low serum sodium might be a reflection of intracellular electrolyte deficit as appears to be the case in tuberculosis (27), the fact that large quantities of water may be retained without salt following paracentesis (28) suggests increased ADH activity. The rising hematocrit and manifestations of circulatory insufficiency which may appear under these circumstances (28) are further indications of a shrinking plasma volume.

It is also of interest that in decompensated cirrhosis, the urine volume and sodium excretion are generally larger at night when the patients are more recumbent than in the daytime (29). This is of particular note since the normal subject has a smaller water and salt excretion at night than during the day—the mechanism of which has not as yet been satisfactorily explained.

It has been suggested that in the initial stages of heart failure a redistribution of blood to the central portions of the vascular bed will necessarily be attended by a deficit of blood and extravascular fluid in the periphery, and presumably in the cranial cavity (16). Although it seems unwise to speculate further, it may be noted that in congestive heart failure, as in cirrhosis, the excretion of water and sodium during nocturnal recumbency exceeds the rate which obtains by day (29). Even the patient in severe failure maintained at strict bed rest tends to assume a less vertical position during sleep than when complying with the postural demands of orthopnea during the day.

CONCLUSIONS

1. Hypotonic expansion of the extracellular fluid volume in the recumbent, water-loaded subject leads to an increased urinary excretion of sodium although the filtered load presented to the renal tubules is actually diminished.

2. The failure of a similar expansion of extracellular volume significantly to augment sodium excretion in the seated subject suggests that the locus of action of expanded volume (or some associated factor) is in the cephalad portion of the body.

3. The hypothesis is suggested that the converse of these observations also holds—contraction of extracellular volume (or an associated factor) in the cephalad portion of the body may be a stimulus for sodium retention.

ACKNOWLEDGMENT

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REFERENCES

1. Starling, E. H., and Verney, E. B., The secretion of urine as studied on the isolated kidney. Proc. Roy. Soc. London, Series B., 1925, **97**, 321.
2. Eggleton, M. G., Pappenheimer, J. R., and Winton, F. R., The mechanisms of dilution diuresis in the isolated kidney and the anesthetized dog. J. Physiol., 1940, **98**, 336.
3. Wesson, L. G., Jr., Anslow, W. P., Jr., Raisz, L. G., Bolomey, A. A., and Ladd, M., Effect of sustained expansion of extracellular fluid volume upon filtration rate, renal plasma flow and electrolyte and water excretion in the dog. Am. J. Physiol., 1950, **162**, 677.
4. McCance, R. A., Young, W. F., and Black, D. A. K., The secretion of urine during dehydration and rehydration. J. Physiol., 1944, **102**, 415.
5. Peters, J. P., Sodium, water and edema. J. Mt. Sinai Hosp., 1950–51, **17**, 159.
6. Welt, L. G., and Orloff, J., The effects of an increase in plasma volume on the metabolism and excretion of water by normal subjects. J. Clin. Invest., 1951, **30**, 751.
7. Crutchfield, A. J., Jr., and Wood, J. E., Jr., Urine volume and total renal sodium excretion during water diuresis. Ann. Int. Med., 1948, **28**, 28.
8. Seldin, D. W., and Tarail, R., Effect of hypertonic solutions on metabolism and excretion of electrolytes. Am. J. Physiol., 1949, **159**, 160.
9. Blomhert, G., Over de Zogenaamde Waterdiurese. Scheltema & Holkema, Amsterdam, 1951.
10. Rosenbaum, J. D., Nelson, W. P., III, Davis, R. K., and Strauss, M. B. Unpublished observations.
11. Meakins, J. C., and Davies, H. W., Observations on the gases in human arterial and venous blood. J. Path. & Bacteriol., 1920, **23**, 451.
12. Strauss, M. B., Davis, R. K., Rosenbaum, J. D., and Rossmeisl, E. C., "Water diuresis" produced during recumbency by the intravenous infusion of isotonic saline solution. J. Clin. Invest., 1951, **30**, 862.
13. Rosenbaum, J. D., Nelson, W. P., III, and Strauss, M. B., The dependence of water diuresis upon electrolyte excretion. J. Clin. Invest., 1950, **29**, 841.
14. Smith, H. W., The Kidney. Structure and Function in Health and Disease. Oxford University Press, New York, 1951.
15. Lewis, J. M., Jr., Buie, R. M., Sevier, S. M., and Harrison, T. R., The effect of posture and of congestion of the head on sodium excretion in normal subjects. Circulation, 1950, **2**, 822.
16. Viar, W. N., Oliver, B. B., Eisenberg, S., Lombardo, T. A., Willis, K., and Harrison, T. R., The effect of posture and of compression of the neck on excretion of electrolytes and glomerular filtration: further studies. Circulation, 1951, **3**, 105.
17. Peters, J. P., Welt, L. G., Sims, E. A. H., Orloff, J., and Needham, J., A salt-wasting syndrome associated with cerebral disease. Tr. A. Am. Physicians, 1950, **53**, 57.
18. Allott, E. N., Sodium and chlorine retention without renal disease. Lancet, 1939, **1**, 1035.
19. Sweet, W. H., Cotzias, G. C., Seed, J., and Yakovlev, P. I., Gastro-intestinal hemorrhages, hyperglycemia, azotemia, hyperchloremia and hypernatremia following lesions of the frontal lobe in man. A. Research Nerv. & Ment. Dis., Proc., 1948, **27**, 795.
20. MacCarty, C. S., and Cooper, I. S., Neurologic and metabolic effects of bilateral ligation of the anterior cerebral arteries in man. Proc. Staff Meetings Mayo Clinic, 1951, **26**, 185.
21. Higgins, G., Lewin, W., O'Brien, J. R. P., and Taylor, W. H., Metabolic disorders in head injury. Hyperchloraemia and hyperchloruria. Lancet, 1951, **1**, 1295.
22. Luetscher, J. A., Jr., and Blackman, S. S., Jr., Severe injury to kidneys and brain following sulfathiazole administration: high serum sodium and chloride levels and persistent cerebral damage. Ann. Int. Med., 1943, **18**, 741.
23. Lewy, F. H., and Gassmann, F. K., Experiments on the hypothalamic nuclei in the regulation of chloride and sugar metabolism. Am. J. Physiol., 1935, **112**, 504.
24. Combined Staff Clinic (College of Physicians and Surgeons, Columbia University), Mechanisms of ascites formation. Am. J. Med., 1950, **9**, 102.
25. Eisenmenger, W. J., Blondheim, S. H., Bongiovanni, A. M., and Kunkel, H. G., Electrolyte studies on patients with cirrhosis of the liver. J. Clin. Invest., 1950, **29**, 1491.
26. Ralli, E. P., Robson, J. S., Clarke, D., and Hoagland, C. L., Factors influencing ascites in patients with cirrhosis of the liver. J. Clin. Invest., 1945, **24**, 316.
27. Sims, E. A. H., Welt, L. G., Orloff, J., and Needham, J. W., Asymptomatic hyponatremia in pulmonary tuberculosis. J. Clin. Invest., 1950, **29**, 1545.
28. Nelson, W. P., III., Rosenbaum, J. D., and Strauss, M. B., Hyponatremia in hepatic cirrhosis following paracentesis. J. Clin. Invest., 1951, **30**, 738.
29. Borst, J. G. G., and de Vries, L. A., The three types of "natural" diuresis. Lancet, 1950, **2**, 1.

EFFECT OF GLYCYRRHIZINIC ACID ON THE ELECTROLYTE METABOLISM IN ADDISON'S DISEASE

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The crude watery extract of the roots of *Glycyrrhiza glabra*, commonly called licorice, has been proved to produce sodium and chloride retention and increased potassium excretion in normal individuals (1) and in a patient with Addison's disease (2). The typical taste of licorice is due to the presence of glycyrrhizin, the ammonium salt of glycyrrhizinic acid. The substance was shown by Borst and his coworkers (1) to be the active principle responsible for the effect of licorice extract on the electrolyte metabolism in normal individuals. In this paper we wish to present the results of electrolyte balance studies in a patient with Addison's disease during treatment with ammonium glycyrrhizinate.

CASE REPORT

A 44-year-old typewriter engineer was admitted in April 1946 for the first time. He gave a history of mild attacks of pulmonary tuberculosis with pleurisy when 16, 20, and 25 years old. Since then he had remained symptom-free until a year before admission when he had noticed fatigue with occasional vomiting and diarrhea. He had developed an aversion for milk but an increased appetite for salted foods. It had been noticed that his skin had become much darker.

On clinical examination he appeared in a moderate nutritional state. There was marked pigmentation of the skin and several dark spots were seen on the oral mucous membrane. There was also an old bilateral fibrous pulmonary lesion. The blood pressure was 100/60. The water excretion test of Robinson, Power, and Kepler (3) showed a disturbance of water excretion. The blood sugar tolerance curve after 50 grams of glucose by mouth did not rise above 122 mg.%.

A diagnosis of Addison's disease was made; the patient was treated with desoxycorticosterone acetate (DCA) and was discharged in a satisfactory condition after one month of observation.

He then remained under ambulatory control on a daily maintenance dosage of 3 mg. DCA, intramuscularly. On our request he appeared willing to be readmitted for a study of the effect of licorice and its components on his condition.

He was readmitted February 28, 1951. The clinical findings were unchanged, but his blood pressure was now 150/70. A "Thorn test" (4) after injection of 25 mg. ACTH showed no effect on eosinophils and only a slight drop in lymphocytes, which was in agreement with the previous diagnosis. The patient was placed on a balance diet and bed rest. Probably as a result, the blood pressure dropped somewhat. During the ensuing weeks it fluctuated between 115 and 140 systolic, and 70 and 90 diastolic.

BALANCE STUDIES

After the patient had had an opportunity to get acclimatized, balance studies were started on March 8, 1951. The results are presented in Figures 1–3. For the first three days he appeared to be in marked positive sodium and chloride balance, so that the administration of DCA was reduced to 2.5 mg. per day. For the next six days the patient still remained in a somewhat positive sodium and chloride balance; the potassium balance was practically in equilibrium.

During the next period a daily amount of 2 grams of ammonium glycyrrhizinate was given by mouth, while the patient received his ordinary injection of DCA.[6] In the next three days there appeared to be an increase in sodium and chloride retention, and the body weight increased from 64 to 65.3 Kg. During the next period of three days DCA was reduced to 2 mg. without appreciable influence on the sodium, chloride or potassium balance. Again three days later, DCA was

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[6] Ammonium glycyrrhizinate 20, added water 300, 2 × 15 cc. per day.

reduced to 1 mg. daily, while the amount of glycyrrhizinate was increased to 3.3 grams per day. Sodium and chloride balances now seemed to approach equilibrium, which encouraged us during the next period to increase the amount of ammonium glycyrrhizinate to 3.6 grams daily and at the same time to stop the administration of DCA completely. When this was done, sodium and chloride balances remained slightly positive while the potassium balance was slightly negative. This condition was maintained for six days. A further increase of the amount of glycyrrhizinate to 4 grams for another three days had no additive effect.

The preparation of ammonium glycyrrhizinate which we had used until then was a brown, impure substance which had been obtained commercially. We now had the opportunity to administer to this patient a preparation of potassium glycyrrhizinate which had been prepared by Dr. de Jong, pharmacist to the Binnengasthuis, and which had been shown by Dr. Borst and his group to produce sodium and chloride retention and increase potassium excretion in a normal in-

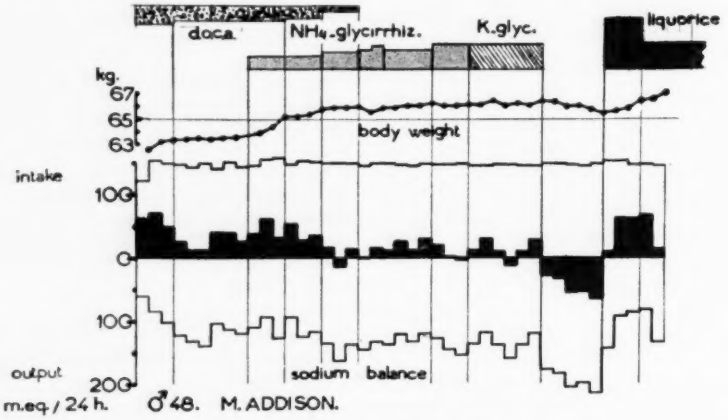

FIG. 1. SODIUM BALANCE PER DAY

Sodium intake is represented above the zero line, urinary output below the line. The difference is represented in black either above (positive balance) or below (negative balance) the line.

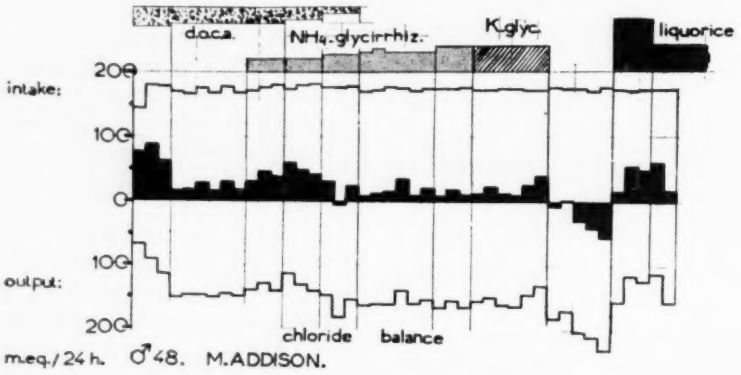

FIG. 2. CHLORIDE BALANCE

(See legend to Figure 1 for explanation)

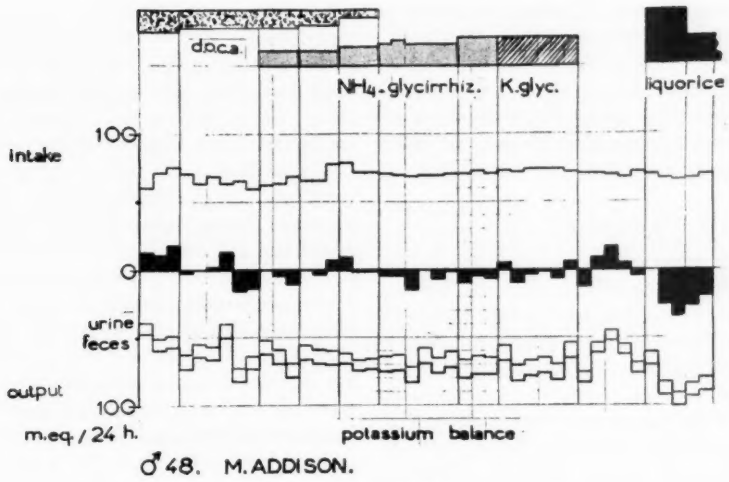

FIG. 3. POTASSIUM BALANCE
(See legend to Figure 1 for explanation)

dividual.[7] This substance was still impure certainly, but it was almost white in color and doubtless considerably purer than the preparation used previously.

During the following period, then, the patient received 4 grams of this reprecipitated potassium glycyrrhizinate, redissolved for this purpose by the addition of ammonia. The slightly positive chloride and sodium balances and slightly negative potassium balance were maintained on this preparation. All this time the patient's weight remained constant and regular chemical analyses of the serum revealed normal values for the urea, sodium, potassium, chloride, bicarbonate, hemoglobin, and serum proteins. Thus, this patient with Addison's disease was kept in electrolyte equilibrium and in satisfactory clinical condition for 15 days with no other treatment than the oral administration of the ammonium or potassium salt of glycyrrhizinic acid.

In the next period the administration of glycyrrhizinate was stopped. The sodium balance reacted immediately by a sharply increased excretion and the chloride balance dropped also, but became negative only two days later. The potassium balance reacted one day later and reversed into a clearcut retention. The body weight decreased and the patient lost 1 Kg. during the next five days.

His clinical condition also deteriorated. He had vague abdominal complaints, experienced fatigue and nausea, and had a sallow complexion. At the end of five days the serum potassium and serum sodium were still normal, but the hemoglobin concentration which had before fluctuated between 11 and 12.6 grams %, had risen to 13.3 grams % as a first indication of hemoconcentration. The patient, who had been very cooperative until now, began to ask for treatment and as the balance studies had definitely shown that the electrolyte equilibrium became grossly disturbed after stopping the glycyrrhizinate administration, we felt that we were not justified in prolonging this experiment.

On March 18th the patient was given crude licorice extract, 40 grams per day. The sodium and chloride balances reacted immediately and the next day there was a marked retention of these electrolytes. The potassium balance also reacted, but again not until one day later. The body weight increased by 1.3 Kg. during the next three days.

After three days it was considered better to reduce the amount of licorice to 20 grams per day. With this treatment the patient remained in good condition; he was discharged five days later and

[7] We are indebted to Dr. de Jong and Dr. Borst for their cooperation in this work.

is at present back at work. His blood chemistry is normal, his blood pressure within normal limits, and he maintains that he feels even better than during the administration of DCA. At the time of writing (June 1st), almost three months after the DCA medication was discontinued, his satisfactory condition continues.

DISCUSSION

The present observation seems to prove that glycyrrhizinic acid, through its action on electrolyte metabolism, can replace DCA for the treatment of Addison's disease. Borst's observation that a licorice extract, from which glycyrrhizinic acid has been precipitated, loses its activity, together with the findings in the above case, offers evidence that glycyrrhizinic acid is indeed the active principle through which crude extract of licorice exerts its action. In contrast to the activity of the crude licorice extract, which took a few days before it manifested itself and which seemed to last for a few days after licorice was withdrawn (2), glycyrrhizinic acid manifested its action on sodium and chloride metabolism promptly; the effect on potassium excretion or retention showed a lag of one day.

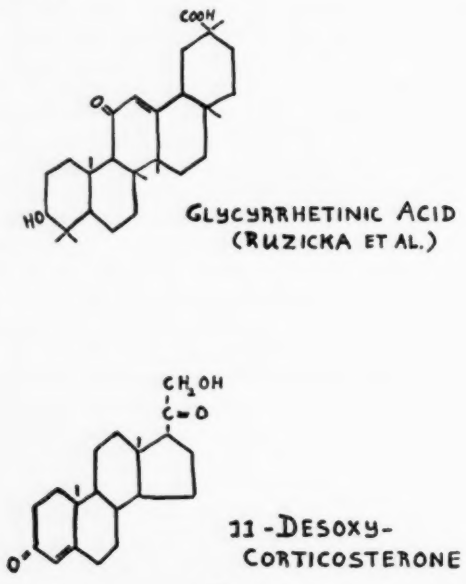

FIG. 4

We have to admit that the best preparation of glycyrrhizinic acid which was used in this investigation was still impure. But it had been reprecipitated four times without appreciable loss of activity which makes the chances considerably smaller that some impurity might be operative.

Glycyrrhizinic acid is a combination of glucuronic acid and glycyrrhetinic acid. The chemical nature of this substance has been investigated by Bergmann (5) and Ruzicka and Leuenberger (6, 7). The structural formula is given in Figure 4. It is a polyterpene compound (more specifically a Triterpenoid) a part of which, through the presence of one OH group and one O-atom in position 11, suggests a certain resemblance to the phenantrene part of the steroid series, to which desoxycorticosterone belongs. It would be a mere speculation to ascribe the similarity in physiological action to this partial resemblance in structure, although this is a point well worth following up. It should not be forgotten, however, that even if the physiological action is the same, the amount of glycyrrhizinic acid, given by mouth in this case, was about a thousand times the quantity of desoxycorticosterone, given by injection, which showed the same activity. To what extent this difference is caused by the different route of administration and poor absorption is another question for further investigation.

SUMMARY

A case of Addison's disease is described in which, after the administration of desoxycorticosterone was stopped, electrolyte equilibrium was maintained by the daily administration of 3–4 grams of ammonium glycyrrhizinate by mouth. On withdrawal of the glycyrrhizinate administration the patient responded by sodium and chloride loss, potassium retention, and clinical signs of dehydration and hemoconcentration. Administration of crude licorice extract brought the electrolyte metabolism back to normal. This observation seems to prove that glycyrrhizinic acid is the active principle through which crude extract of licorice exerts its influence on the electrolyte metabolism in Addison's disease.

REFERENCES

1. Molhuysen, J. A., Gerbrandy, J., de Vries, L. A., de Jong, J. C., Lenstra, J. B., Turner, K. P., and

Borst, J. G. G., A liquorice extract with desoxycortone-like action. Lancet, 1950, **2**, 381.

2. Groen, J., Pelser, H., Willebrands, A. F., and Kamminga, C. E., Extract of licorice for the treatment of Addison's disease. New England J. Med., 1951, **244**, 471.
3. Robinson, J. F., Power, M. H., and Kepler, E. J., Two new procedures to assist in the recognition and exclusion of Addison's disease; preliminary report. Proc. Staff Meet. Mayo Clinic, 1941, **16**, 577.
4. Thorn, G. W., Forsham, P. H., Prunty, F. T. G., and Hills, A. G., Test for adrenal cortical insufficiency; response to pituitary adrenocorticotropic hormone. J. A. M. A., 1948, **137**, 1005.
5. Bergmann, F., Uber das enzymatische Verhalten von Glucuroniden und über Glycyrrhizinsäure. Biochem. Ztschr., 1933, **267**, 296.
6. Ruzicka, L., and Leuenberger, H., Zur Kenntniss der Glycyrrhetinsäure. Helvet. chim. Acta, 1936, **19**, 1402.
7. Ruzicka, L., Zur Kenntniss der Glycyrrhetinsäure. Helvet. chim. Acta, 1943, **26**, 2278.

CHANGES IN THE ACID–BASE EQUILIBRIUM IN ESSENTIAL HYPERTENSION

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In a study of a group of hospitalized patients in whom there was no reason to suspect a disturbed acid-base balance, it was found that a few patients had low blood pH values. The only factor in common to these patients with the low pH values was that all had essential hypertension. It has been demonstrated in human subjects that experimental hypertension can be produced with desoxycorticosterone when sodium chloride is administered simultaneously (1); that there is a relationship between the ratio of sodium to chloride in essential hypertension and the severity of the hypertension (2); and that the restriction of sodium chloride has a beneficial effect in the treatment of patients with essential hypertension (3). This evidence of changes in ionic equilibrium of the blood in patients with essential hypertension prompted further investigation to determine whether an increased hydrogen ion concentration of the arterial blood was common in this disease.

MATERIAL AND METHODS

A group of 49 patients was studied, consisting of 14 normotensive patients, 19 essential hypertensive patients with normal kidney function and 16 hypertensive patients with renal functional impairment. In the last group of patients the renal impairment was due to glomerulonephritis in two cases and secondary to essential hypertension in the remaining ones. In order to be classed as hypertensive the patients had to have a blood pressure exceeding 150 mm. of Hg systolic and 90 mm. of Hg diastolic after hospitalization for one week. The classification of normal kidney function was on a clinical basis. The clinical criteria used were a specific gravity of 1,020 or better in the morning sample and no evidence of nitrogen retention as revealed by a patient's blood urea nitrogen below 15 mg. per 100 cc. All of these patients were routine hospital admissions who were admitted for some condition other than hypertension. Patients were selected who were free of congestive failure and any pathological condition which might interfere with normal pulmonary ventilation. In addition, no patients were studied who were receiving any drug known to change the acid-base pattern. All patients were prepared as for measurement of the basal metabolic rate.

Samples of arterial and venous blood were obtained with a minimum of inconvenience to the patient in order to prevent emotional excitement and possible hyperventilation. In a few patients it was impossible to obtain both samples without exciting the patient or for some technical reasons, and therefore determinations on either arterial or venous blood are omitted. The arterial blood was drawn from the brachial artery into an oiled Luer-Lok syringe containing a small amount of neutral heparin and sodium fluoride and sealed with a soldered needle hub. Venous blood was obtained without stasis from a vein of the same arm. In order to ensure representative samples, 10 ml. portions of blood were collected in periods of one to one and one-half minutes.

All determinations of the pH were performed in triplicate at room temperature with a Cambridge pH meter using a standardized (Taylor) buffer solution with a pH of 7.38 as a reference solution. The blood samples were kept on ice until shortly before being used for the pH determination at which time they were warmed to room temperature by immersion into a water bath. Oxygen content, oxygen capacity and carbon dioxide content were determined on whole blood, in duplicate, by the method of Van Slyke and Neill (4). Hematocrits were determined by the conventional Wintrobe method. The partial pressures of carbon dioxide were calculated from the experimental data, using the nomograms of Van Slyke and Sendroy (5).

RESULTS

In Table I are presented the results of examination of 14 normotensive patients. It will be noted that in none of these patients is the arterial pH below 7.38 or does the carbon dioxide tension exceed 46 mm. of Hg. The mean pH of this group is 7.41, the mean carbon dioxide tension 39.6 mm. of Hg. The last patient (S. H.) had acute glomerulonephritis without hypertension and exhibited a normal venous blood pH. (The arterial pH was not determined.) The figures in this table are not intended to provide conclusive statistical data of normals but are to serve as controls and as a check of the validity of the methods and techniques used.

TABLE I

Results of studies on normotensive patients

Patient's initials	Age	pH arterial blood	pH venous blood	CO_2 content arterial blood	Oxygen capacity (C) or Hematocrit (H)	pCO_2 arterial plasma (calculated)
	yrs.	units	units	volumes per cent	vol. per cent (C) or per cent (H)	mm. of Hg
J. McK.	64	7.39		57.85	34.0 (H)	46
M. W.	53	7.40		47.90	17.35 (C)	42
H. L.	54	7.44		44.77	18.17 (C)	35
A. L.	45	7.39		44.78	14.78 (C)	37
I. M.	62	7.40		46.72	22.04 (C)	40
R. M.	51	7.39		49.20	21.17 (C)	43
P. B.	61	7.48		52.64	21.85 (C)	39
J. G.	53	7.51		44.59	20.17 (C)	31
M. B.	59	7.41	7.38	48.46	17.95 (C)	40
A. U.	60	7.38	7.37	49.94	41.0 (H)	44
R. M.	52	7.40	7.37	42.48	32.0 (H)	34
E. D. M.	65	7.39	7.37	49.26	43.0 (H)	42
E. D.	46	7.41		51.05	21.66 (C)	42
S. H. *	38		7.39	(55.18 venous)	46.0 (H)	
Mean	54.5	7.41	7.38	48.43		39.6

* Patient with acute glomerulonephritis

TABLE II

Results of studies on patients with essential hypertension without clinical evidence of renal impairment

Patient	Age	pH arterial blood	pH venous blood	CO_2 content arterial blood	Oxygen capacity (C) or Hematocrit (H)	pCO_2 arterial plasma (calculated)
	yrs.	units	units	volumes per cent	vol. per cent (C) or per cent (H)	mm. of Hg
T. K.	50	7.35			18.72 (C)	
A. D.	51		7.31	52.40	15.95 (C)	50
H. E.	60	7.35		52.64	17.65 (C)	49
F. O.	47	7.33		52.36	22.27 (C)	53
I. R.	59	7.34	7.31	49.40	17.50 (C)	46
D. L.	60	7.36		51.70	16.50 (C)	47
R. B.	49		7.26		18.16 (C)	
J. M.	43	7.34	7.29	51.78	36.5 (H)	52
A. W.	59	7.38	7.32	50.48	18.15 (C)	46
L. H.	56	7.34	7.27	46.30	31.0 (H)	43
C. R.*	28	7.48	7.47	48.97	22.60 (C)	35
M. P.	47	7.36	7.33	49.00	27.0 (H)	43
K. H.	58	7.35		55.23	38.5 (H)	52
C. E.	51	7.36	7.29	49.16	41.0 (H)	47
J. A.	45	7.34		48.70	40.0 (H)	48
P. B.	62	7.35	7.33	55.87	38.0 (H)	52
C. B.	56	7.37	7.34	51.85	40.0 (H)	48
J. P.	59	7.35	7.32	46.55	45.0 (H)	46
C. Y.	61	7.35	7.32	55.30	41.5 (H)	49
Mean	52.6	7.36	7.32	51.04		47.4

*Patient with suspected pheochromocytoma

The results of the studies on patients with essential hypertension and normal kidney function are presented in Table II. In this group, with one exception, the arterial pH values range between 7.33 and 7.38, and the arterial carbon dioxide partial pressure varies between 43 and 53 mm. of Hg with a mean of 47.4. The mean pH of this group is 7.36. This represents a 12 per cent increase in hydrogen ion concentration in the hypertensive group. The differences in pH between normotensive and hypertensive patients of this group, although slight, are consistent and exceed a probability of 0.01. One patient (C. R.) does not exhibit these changes. This patient was suspected, clinically, of having a pheochromocytoma. Although the patient died, an autopsy could not be performed and the diagnosis was never substantiated; therefore this case has been included with the group having essential hypertension.

Patients with hypertension and renal impairment, listed in Table III, also have low arterial pH values ranging from 7.23 to 7.37, but the carbon dioxide tensions are normal or below normal with a mean of 39.1 mm. of Hg. The mean arterial pH of this group is 7.31. The first two patients listed in the table have hypertension secondary to chronic glomerulonephritis; three patients (L. B., G. J., M. J.) have the malignant form of essential hypertension. The remaining patients have prolonged hypertension with secondary renal functional impairment of varying degrees, as evidenced by variations in the ability to concentrate urine and variations in degree of nitrogen retention.

DISCUSSION

For some time, the range of blood pH from about 7.30 to 7.50 was accepted as normal (6). Recent studies (7, 8) indicate a narrower range of normal values from 7.37 to 7.45. This change is due to refinements in the technique for measuring hydrogen ion concentration which were not available to the earlier investigators, and also to the elucidation of many factors which were sources of error in collecting and handling of the blood prior to the determination. Other factors which helped to account for the previously accepted wide spread of normal are not within the scope of the present paper but some of them will be mentioned

TABLE III

Results of studies on patients with hypertension and renal functional impairment

Patient's initials	Age	pH arterial blood	pH venous blood	CO_2 content arterial blood	Oxygen capacity (C) or Hematocrit (H)	pCO_2 arterial plasma (calculated)
	yrs.	units	units	volumes per cent	vol. per cent (C) or per cent (H)	mm. of Hg
F. M.*	46	7.33	7.32	38.04	14.00 (C)	36
W. M. H.*	50	7.26	7.23	24.20	22.5 (H)	26
J. G.	47	7.32		49.91	21.13 (C)	51
C. S.	63		7.33		30.0 (H)	
V. A.	64	7.35		46.65	13.89 (C)	41
J. D. D.	62	7.35	7.33	50.78	16.20 (C)	47
S. T.	45	7.34	7.33	46.10	13.41 (C)	41
R. R.	54	7.29	7.21	31.20	22.30 (C)	34
S. E.	56	7.36	7.30	41.76	18.05 (C)	38
S. B.	59	7.33	7.31	44.27	35.0 (H)	42
B. McM.	53	7.37	7.35	44.50	31.0 (H)	39
L. B. **	36	7.25	7.23	35.43	25.5 (H)	39
G. J. **	34	7.27	7.22	29.30	29.0 (H)	30
E. B.	64	7.32	7.30	45.25	31.5 (H)	44
H. H.	65	7.30		43.61	31.0 (H)	44
M. J. **	41	7.23		29.98	25.0 (H)	34
Mean	52.4	7.31	7.28	39.93		39.1

* Patients with hypertension secondary to glomerulonephritis

** Patients with the malignant form of essential hypertension

TABLE IV

Statistical significance of differences between groups

	Groups 1 and 2		Groups 2 and 3		Groups 1 and 3	
	t score	p	t score	p	t score	p
pH of arterial blood	3.70	>0.01	3.65	>0.01	6.45	>0.01
pH of venous blood	4.12	>0.01	3.4	>0.01	4.02	>0.01
CO_2 content of arterial blood	2.04	0.05	4.60	>0.01	2.92	>0.01
pCO_2 of arterial plasma (calc.)	4.72	>0.01	4.23	>0.01	0.229	<0.50

Group 1: Normotensive patients.
Group 2: Patients with essential hypertension without clinical evidence of renal impairment.
Group 3: Hypertensive patients with renal functional impairment.

in explanation of the procedure used in this study.

Study of the patients under basal conditions eliminated some of these sources of error since the diet or degree of activity prior to obtaining the blood sample may affect the acid-base pattern slightly (7). Hospitalizing the patients for one week prior to study definitely established the diagnosis of sustained hypertension and allowed them to become accustomed to the procedure of venipuncture. This tended to eliminate any excitement with resulting increase in ventilation which would have lowered the carbon dioxide tension and raised the pH of the blood, with a tendency to obscure the abnormalities found in this study.

The differences in pH and carbon dioxide tensions between the normotensive and the hypertensive group with normal kidney function cannot be explained by the present study. They are, nonetheless, of definite statistical significance (Table IV). They cannot be due to differences in age groups since the average age and age distribution are nearly identical in all three groups. It has been shown (9) that the pH of the arterial blood progressively decreases with age but it is not below 7.38 at the age of 65. The differences may be due to early changes in renal physiology because the classification of these patients as having normal kidney function is based on the clinical evidence of no nitrogen retention and the ability to concentrate the urine. Goldring and Chasis (10) have shown that there are changes in renal physiology which precede changes in these two functions of the kidney.

The patients in the group of essential hypertension with normal kidney function presented an increased partial pressure of carbon dioxide accompanying the increased blood acidity. This is the pattern of primary respiratory acidosis. The increased acidity of the arterial blood with a decrease in the partial pressure of carbon dioxide in patients with hypertension and renal impairment is the typical picture of metabolic acidosis, whereby fixed acids accumulate in the blood with a consequent lowering of the carbon dioxide tension due to respiratory compensations. In our patients with renal impairment secondary to essential hypertension the carbon dioxide tension is not as low as predicted by the formula of Gray (11). In the present study the difference in partial pressure of carbon dioxide in arterial plasma between the normotensive group and the group of hypertensives with renal impairment is not statistically significant (Table IV).

SUMMARY

Evidence is presented that patients with essential hypertension and normal kidney function have a higher hydrogen ion concentration and a higher partial pressure of carbon dioxide in the arterial blood than normotensive individuals. In

the normotensive group the pH was found to be 7.38 or higher, with a mean of 7.41, while the hypertensive patients have a pH of the arterial blood of 7.38 or below, with a mean of 7.36. This increased acidity of the blood in patients with hypertension and normal kidney function is accompanied by an increased carbon dioxide tension which averages 47.4 mm. of Hg, compared to 39.6 mm. in the normotensive group. When renal impairment secondary to essential hypertension occurs, the acid-base pattern approaches that of metabolic acidosis.

REFERENCES

1. Perera, G. A., and Blood, D. W., Pressor activity of desoxycorticosterone acetate in normotensive and hypertensive subjects. Ann. Int. Med., 1947, **27**, 401.
2. Selye, F. L., Biochemical changes in hypertension. Canad. M. A. J., 1947, **57**, 325.
3. Grollman, A., Harrison, T. R., Mason, M. F., Baxter, J., Crampton, J., and Reichsman, F., Sodium restriction in the diet for hypertension. J. A. M. A., 1945, **129**, 533.
4. Van Slyke, D. D., and Neill, J. M., The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. J. Biol. Chem., 1924, **61**, 523.
5. Van Slyke, D. D., and Sendroy, J., Jr., Studies of gas and electrolyte equilibria in blood; line charts for graphic calculations by the Henderson-Hasselbalch equation, and for calculating plasma carbon dioxide content from whole blood content. J. Biol. Chem., 1928, **79**, 781.
6. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Vol. I. Interpretations. Williams & Wilkins Co., Baltimore, 1931, pp. 936, 940, and 941.
7. Shock, N. W., and Hastings, A. B., Studies of the acid-base balance of the blood. III. Variations in the acid-base balance of the blood in normal individuals. J. Biol. Chem., 1934, **104**, 585.
8. Wilson, R. H., The pH of whole arterial blood. J. Lab. & Clin. Med., 1951, **37**, 129.
9. Shock, N. W., and Yiengst, M. J., Age changes in the acid-base equilibrium of the blood of males. Gerontology, 1950, **5**, 1.
10. Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease. The Commonwealth Fund, New York, 1944.
11. Gray, J. S., Pulmonary Ventilation and its Physiological Regulation. Charles C Thomas, Springfield, Ill., 1950, p. 37.

EVIDENCE FOR THE PRESENCE OF INSULIN IN BLOOD SERUM. A METHOD FOR AN APPROXIMATE DETERMINATION OF THE INSULIN CONTENT OF BLOOD[1]

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In recent years the carbohydrate metabolism of the isolated rat diaphragm has been widely studied (1–8). Using this preparation, Gemmill (2, 3) succeeded in demonstrating *in vitro* the stimulating effect of insulin on the glucose utilization and glycogen synthesis of an isolated muscle. It has since been shown that a quantitative relationship exists between the amount of insulin present in the incubation medium and its effect on the glucose metabolism of the diaphragm (5, 6). Furthermore, it was found that the diaphragms of young rats are sensitive to extremely small concentrations of insulin (9). This investigation was intended to determine if the glucose metabolism of the isolated rat diaphragm could be used to demonstrate the presence of insulin in blood serum and to determine the concentration of this hormone in blood.

METHODS

The standard technique used in these experiments is as follows: Four young rats of 80–100 gr. body weight, fasted for 24 hours, are killed by decapitation. The diaphragms are taken out quickly and carefully, to avoid trauma as much as possible, and divided in half. The hemi-diaphragms are washed in ice cold buffer solution (without glucose), and dried between filter paper. Four of the hemi-diaphragms, each from a different rat, are then placed in a small flask; the remaining four are placed in a similar flask. Into each flask, beforehand, have been pipetted 2 ml. of an ice cold buffer solution with a mineral composition closely resembling blood plasma (10), and after equilibration with a 7% CO_2 – 93% O_2 mixture, a pH of 7.4. In addition the buffer contained 200 mg.% glucose. The glucose buffer solution in one of the flasks contained insulin in the concentration to be tested; the other served as control. Following equilibration with the gas mixture of 93% oxygen plus 7% carbon dioxide, both flasks are incubated at 37° with shaking at a rate of 120/minute. After 90 minutes the flasks are removed and cooled. The muscle tissue, having been dried between filter paper, is weighed and the amount of glucose is determined in the medium (11). The difference in glucose utilization per 100 mg. of wet diaphragm between the flask containing insulin and the control flask indicates the effect of the added insulin.

In other experiments, four flasks containing the quarter-diaphragms of eight rats are used. Each diaphragm is cut in four pieces and the four quarters are distributed among the four flasks. Here, each flask contains eight quarter-diaphragms of eight different rats. This procedure is chosen so that the variation in glucose consumption and in sensitivity to insulin between individual diaphragms will be neutralized. The four flask technique enables us to compare the glucose utilization in glucose buffer solutions of three different insulin concentrations with the glucose utilization in a control vessel containing only glucose buffer. Generally speaking, the glucose utilization of hemi-diaphragms in buffer solutions without insulin is lower than that of quarter-diaphragms (on an equal weight basis), but the effect of added insulin is usually more evident when hemi-diaphragms are used. The glucose utilization of the diaphragm increases even further when it is divided into more fragments, but the effect of insulin becomes progressively less. For this reason, pieces of diaphragm smaller than a quarter-size have not been used in this work.

Figure 1, a concentration action curve, illustrates the effect of different concentrations of a sample of purified insulin (expressed as units/ml. of the medium) on the glucose utilization (in mg./100 mg. wet tissue) of the rat diaphragm. The graph illustrates that, as the insulin concentration is increased, the insulin effect increases until, at about 10^{-3} units/ml., it tends to become more or less constant. In the region of lower concentrations, it was found that amounts as low as 5×10^{-4} still produced a significant increase in glucose utilization. The sample of pure insulin used in these experiments contained 28 units/mg.[2] Assuming a molecular weight of insulin of 48,000, a concentration of 5×10^{-4} units/ml. is equal to 4.5×10^{9} molecules per flask. This number of insulin molecules enabled the diaphragms to utilize about 10^{15} more molecules of glucose than diaphragms in the control flask without insulin.

[1] These researches were supported by grants from the Organon Laboratories, Inc., and the Netherlands Diabetics Association.

[2] We are indebted to Dr. J. Lens of Organon Laboratories, Inc. for a gift of pure insulin.

In the course of further studies, it was found that whereas the sensitivity of the insulin-glucose diaphragm system is usually of the same order as given in Figure 1, variations in the sensitivity are occasionally encountered. In addition to the curve of Figure 1, Figure 2 shows the least sensitive concentration action curve. This was obtained in the summer of 1950, when the sensitivity of the diaphragms used was obviously much less, especially in the region of the lower insulin concentrations. The cause of the phenomenon is unknown to us. Other investigators have probably refrained from using the isolated rat diaphragm as a test object for the standardization of insulin because of this variation in sensitivity. It seems to us, however, that occasional evidence of a smaller sensitivity need not prohibit the use of the rat diaphragm for this purpose, providing proper control standardizations are carried out with every experiment.

Demonstration of the presence of insulin activity in normal serum

The procedure for this experiment was the same as that used for the standardization curve with the difference that here, one vessel contained the diaphragm suspended in 2 ml. of a glucose buffer, whereas the other vessel contained 1.68 ml. of buffer to which 0.32 ml. of serum had been added. The initial concentration of glucose in both vessels was adjusted to 200 mg.%. After incubation and shaking for 90 minutes, the amount of glucose utilized in both vessels was determined and expressed as mg./100 mg. of wet tissue. The difference between the glucose utilization in the medium with serum and in the buffer without serum was taken as the "insulin effect" of the serum.

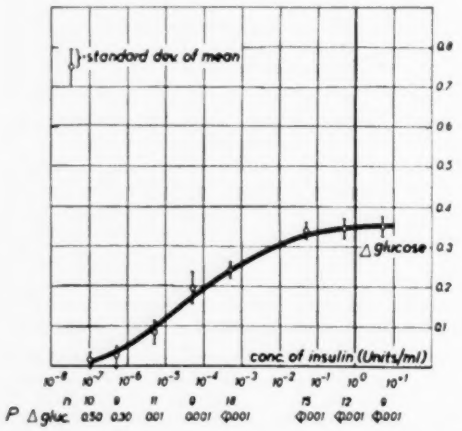

FIG. 1. CONCENTRATION ACTION CURVE, SHOWING THE INCREASE IN GLUCOSE UTILIZATION OF THE ISOLATED RAT DIAPHRAGM BY DIFFERENT CONCENTRATIONS OF INSULIN IN THE MEDIUM

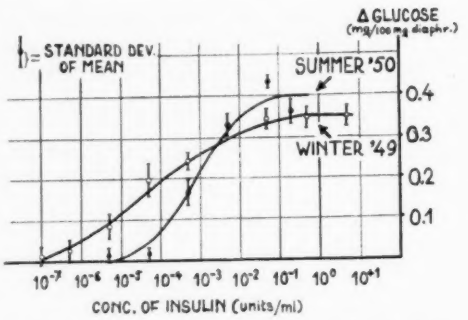

FIG. 2. VARIABILITY OF THE CONCENTRATION ACTION CURVE OF THE QUANTITATIVE EFFECT OF INSULIN UPON THE GLUCOSE UTILIZATION OF THE ISOLATED RAT DIAPHRAGM

RESULTS

Table I gives the results of eight experiments on normal human serum and five on normal dog serum. From these figures it appears that normal serum produces an "insulin effect" in the sense that it produces an extra utilization of glucose of 0.1–0.2 mg./100 mg. of diaphragm after one and one-half hours.

Table II gives the results of similar experiments carried out with the serum of four cases of diabetic coma and the serum of two dogs after pancreatectomy. The first dog (R 75) died in severe coma six days after the operation. The second dog (F 78) was maintained on insulin after the operation. It died in coma 20 days after insulin was withdrawn, and at postmortem examination, only minute amounts of pancreatic tissue were found. It will be seen that all these sera did not significantly increase the glucose utilization of the diaphragm.

Figure 3 shows the results of repeated determinations on the serum of dog F 78 after insulin was withdrawn. A progressive decrease of the insulin effect occurred as the diabetes developed until, on the 20th day, when the dog died in coma, there was almost no insulin content.

Table III gives the results of five experiments carried out with the serum of diabetic patients who were under treatment with insulin. It will be seen that the values obtained were all within the normal range. It would seem that the value from patient S was the lowest of this series because it was obtained with a serum sample taken

TABLE I

Difference in glucose utilization (mg.) by 100 mg. diaphragm in: a) buffer + serum of normal humans or dogs, b) buffer without serum

Serum from	Av. extra glucose utilization (a−b)	Standard dev.	No. of observations
	(mg.)		
K.	+0.171	0.075	3
H.	+0.181	0.092	6
W.	+0.186	0.038	6
G.	+0.131	0.046	6
K.	+0.103	0.050	6
W.	+0.175	0.049	6
K.	+0.159	0.065	5
K.	+0.207	0.060	6
Dog X.	+0.168	0.047	6
Dog R.75	+0.226	0.039	6
Dog F.78	+0.202	0.050	6
Dog S.	+0.182	0.072	6
Dog 105	+0.098	0.049	6

TABLE II

Difference in glucose utilization (mg.) by 100 mg. diaphragm in: a) buffer + serum during diabetic coma, b) control buffer without serum

Serum from	Av. extra glucose utilization (a−b)	Standard dev.	No. of observations
	(mg.)		
Pat. J.	−0.043	0.050	3
Pat. D.	−0.014	0.042	5
Pat. B.	+0.028	0.037	6
Pat. P.	+0.027	0.024	5
Dog R.75*			
2 days without ins.	−0.048	0.067	6
4 days without ins.	0	0.031	6
Dog F.78*			
20 days without ins.	+0.033	0.056	6

* After pancreatectomy.

after insulin had been withheld for 24 hours. However, in other experiments, not recorded here, there was only a small and not always statistically significant difference between the insulin effect of diabetic serum taken before and after insulin was withheld.

Table IVA gives the results of three sets of experiments, all carried out on the serum of patient K, who was admitted with diabetic coma. The technique of these experiments was so arranged that the diaphragms were incubated (a) in buffer with serum obtained after the patient had recovered from coma, and (b) in serum obtained during the coma, before the beginning of treatment.

This procedure was chosen so as to make all other conditions in the two flasks as equal as possible. It will be seen that there was a definitely higher glucose utilization in the medium containing the serum taken after recovery, as compared to that in the diluted coma serum. Table IVB also gives the results of two similar experiments carried out with the sera of dogs, before and after pancreatectomy, showing the same effect.

Figure 4 represents a "time curve" in which the rat diaphragms were incubated (a) in serum

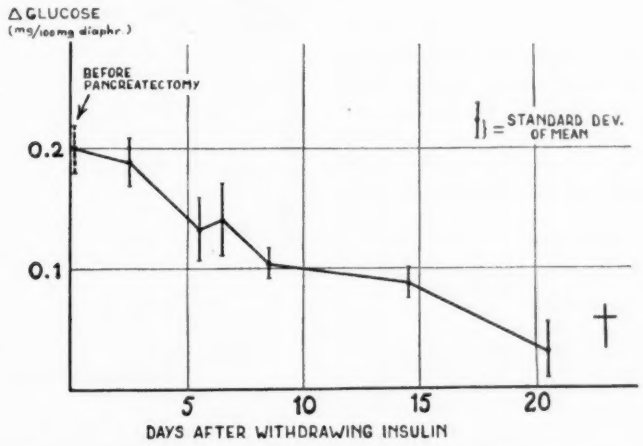

FIG. 3. DIMINUTION OF THE "INSULIN EFFECT" OF THE SERUM OF THE DEPANCREATIZED DOG, TESTED ON THE ISOLATED RAT DIAPHRAGM

TABLE III

Difference in glucose utilization (mg.) by 100 mg. diaphragm in: a) buffer + serum of diabetic patient while under insulin treatment, b) buffer without serum

Serum from	Av. extra glucose utilization (a − b)	Standard dev.	No. of observations
	(mg.)		
J.	+0.207	0.035	3
S.	+0.095	0.053	6
D.	+0.222	0.077	6
H.	+0.124	0.033	6
B.	+0.216	0.059	6

obtained during coma, and (b) in serum obtained after recovery by treatment with insulin. During the incubation, samples were taken from the flasks at regular intervals, and the amounts of glucose compared. It will be seen that the glucose utilization was consistently higher in the "recovery serum" than in the coma serum.

TABLE IV A

Difference in glucose utilization (mg.) by 100 mg. diaphragm in serum from Patient K during coma and after recovery

a = buffer plus serum obtained after recovery
b = buffer plus serum obtained during coma

No. of observations	(a − b)	Standard deviation
	(mg.)	
2	+0.100	0.028
6	+0.067	0.044
6	+0.102	0.041

The above results, summarized in Figure 5, seem to indicate that normal human or dog serum contains a substance which is able to stimulate the utilization of glucose, and which is absent from the serum of patients with diabetic coma or dogs after complete pancreatectomy. The possibility was considered that this difference between normal serum and serum of diabetic coma was due to an inhibition of the glucose utilization by the presence of ketone bodies. *A priori* this did not seem probable as the values obtained with the coma serum were not significantly lower than those obtained when the diaphragms were incubated in buffer without serum. However, in order to investigate the possibility of a toxic effect of the ketone bodies in coma serum on the glucose utilization, the experiments given in Table V were carried out. Each experiment consisted of four

TABLE IV B

Difference in glucose utilization (mg.) by 100 mg. diaphragm in serum from two dogs before and after pancreatectomy

a = buffer plus normal serum
b = buffer plus serum after pancreatectomy

Serum from	No. of observations	(a − b)	Standard deviation
		(mg.)	
Dog R.75	6	+0.159	0.053
Dog F.78	6	+0.146	0.025

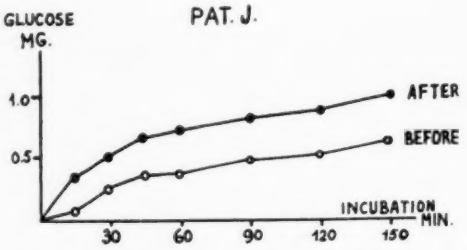

FIG. 4. COMPARISON OF THE GLUCOSE UTILIZATION OF THE ISOLATED RAT DIAPHRAGM INCUBATED IN SERA OBTAINED FROM ONE PATIENT BEFORE AND AFTER INSULIN TREATMENT FOR DIABETIC COMA

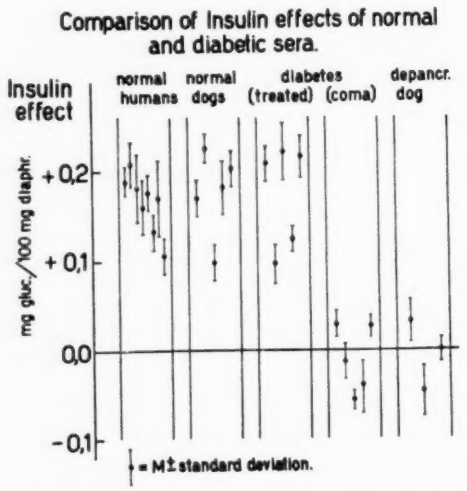

FIG. 5. SUMMARY OF THE DATA ILLUSTRATING THE DIFFERENCE IN "INSULIN EFFECT" BETWEEN NORMAL SERA AND SERA FROM PATIENTS OR DOGS IN UNTREATED DIABETIC COMA

TABLE V

Influence of acetone and β-hydroxybutyric acid on the glucose utilization (mg.) by 100 mg. diaphragm

	Medium	Acetone	β-hydroxy butyric ac.	Av. glucose util. (*mg./100 mg. tissue*)	"Insulin" effect of serum	No. of observations
I	a) 2 ml. buffer	−	−	0.534		6
	b) 0.32 ml. serum + 1.68 ml. buffer	−	−	0.590	0.056±0.062	6
	c) 2 ml. buffer	+	+	0.532		6
	d) 0.32 ml. serum + 1.68 ml. buffer	+	+	0.612	0.080±0.049	6
II	a) 2 ml. buffer	−	−	0.571		6
	b) 0.32 ml. serum + 1.68 ml. buffer	−	−	0.669	0.098±0.049	6
	c) 0.32 ml. serum + 1.68 ml. buffer	+	+	0.683	0.112±0.089	6
III	a) 2 ml. buffer	−	−	0.694		6
	b) 0.32 ml. serum + 1.68 ml. buffer	−	−	0.786	0.092±0.065	6
	c) 2 ml. buffer	+	+	0.691		6
	d) 0.32 ml. serum + 1.68 ml. buffer	+	+	0.748	0.057±0.024	6

sets of observations carried out on diaphragms suspended (a) in buffer solution, (b) in buffer solution to which serum had been added, (c) in buffer with added acetone and β hydroxybutyric acid, both in a concentration of 30 mg.%, and (d) in buffer with serum and the same concentration of ketone bodies. If the difference observed between the "insulin activity" of normal and coma serum was due to the presence of ketone bodies, one would expect that the insulin activity of normal serum would disappear when acetone and β hydroxybutyric acid were added. It was found that this addition had no influence on the glucose utilization of the diaphragm, and did not diminish the "insulin effect" of normal serum.

A concentration of both ketone bodies of 30 mg. % in the medium in these experiments corresponds to a concentration in the undiluted serum of 200 mg.% which is about the concentration one may find in the serum during diabetic coma.

Table VI gives the results of two experiments in which insulin in concentrations of 10^{-2} and 10^{-4} units/ml. were added to coma serum. This was done to test the possibility that the coma serum might contain substances which inhibited the normal insulin effect. The effect of added insulin was apparently not influenced by the presence of the serum. Thus it appears that the absence of an insulin effect in the coma serum is not so much due to the presence of substances that counteract or inhibit insulin activity, but to the absence of insulin itself.

TABLE VI

Effect of added insulin on glucose utilization (mg.) by 100 mg. diaphragm in buffer + serum

	Medium	Added insulin (*U./ml.*)	Glucose utilization (*mg./100 mg. tissue*)	"Insulin" effect	No. of observations	Remarks
I	a) 2 ml. buffer	−	0.627	−	5	
	b) 0.24 ml. serum + 1.76 ml. buffer	−	0.613	−0.014±0.042	5	coma-serum
	c) 2 ml. buffer	10^{-2}	0.770	+0.143±0.053	5	
	d) 0.24 ml. serum + 1.76 ml. buffer	10^{-2}	0.769	+0.142±0.047	5	
II	a) 0.265 ml. serum + 1.735 ml. buffer	−	0.586	−	6	coma-serum
	b) 0.265 ml. serum + 1.735 ml. buffer	10^{-4}	0.689	0.103±0.032	6	
III	a) 2 ml. buffer	−	0.813	−	6	
	b) 0.32 ml. serum + 1.68 ml. buffer	−	0.863	+0.050±0.042	6	coma-serum
	c) 2 ml. buffer	10^{-2}	1.031	+0.218±0.031	6	
	d) 0.32 ml. serum + 1.68 ml. buffer	10^{-2}	1.011	+0.198±0.030	6	

Influence of serum on glycogen synthesis

Gemmill (2, 3), Stadie and Zapp (5), and Tuerkischer and Wertheimer (7) demonstrated that insulin not only increases glucose utilization of the isolated rat diaphragm, but also promotes glycogen synthesis. If the substance which is present in normal serum and stimulates the utilization of glucose is really insulin, it should also have a positive influence on glycogen synthesis. This actually proved to be the case. Table VII gives the results of five experiments, three on normal serum and two on serum of diabetic patients under insulin treatment. In all cases there was a significant increase in glycogen synthesis in comparison to the flasks in which the diaphragms were incubated in buffer without serum.

TABLE VII

"Insulin effect" of serum on glucose utilization and on glycogen synthesis

Experiment	Extra glucose utilized (*mg./100 mg. diaphragm tissue*)		Extra glycogen synthesized (*mg./100 mg. diaphragm tissue*)		No. of observations
1	0.092	±0.026	0.086	±0.013	6
2	0.207	±0.024	0.171	±0.020	5
3	0.183	±0.014	0.072	±0.028	6
4*	0.157	±0.014	0.097	±0.015	6
5*	0.124	±0.013	0.121	±0.008	6

* Diabetic sera.

Inhibition of insulin effect of serum by addition of cysteine or glutathione

Insulin loses its activity in the presence of SH compounds such as cysteine or glutathione. Table VIII gives the results of experiments which show this inactivation by cysteine or glutathione on the isolated rat diaphragm. It will be seen that the effect of both pure insulin and serum either disappeared or diminished considerably when the insulin or serum had been treated with one of these SH compounds. The results of these experiments are a further proof that the stimulating effect of the serum on the glucose utilization of the isolated rat diaphragm is indeed due to the presence of insulin contained therein.

Approximate estimation of the concentration of insulin in normal human serum

The figures given in Table I for the insulin effect of normal serum cannot be used for a computation of the actual insulin content. Some of these figures were obtained before we were aware of the variability in the sensitivity of the diaphragm in the lower range of the concentration action curve. For this reason, in further ex-

TABLE VIII

Inhibition of the effect of insulin or serum on glucose utilization of the isolated rat diaphragm by cysteine or glutathione

Exp. no.	Medium (2 ml. per flask)						Glucose utilization (*mg./100 mg. tissue*)	
	Buffer	Insulin	Serum	Cysteine	Glutathione	Init. gluc. conc.	Average utiliz.	Effect of insulin or serum
	(*ml.*)	(*U./ml.*)	(*ml.*)					
I a	2	–	–	–	–	200	0.628	
b	2	10^{-3}	–	–	–	200	0.826	b−a = 0.198±0.026
c	2	–	–	+	–	200	0.644	
d	2	10^{-3}	–	+	–	200	0.658	d−c = 0.014±0.013
II a	2	–	–	–	–	175	0.555	
b	1.73	–	0.27	–	–	175	0.639	b−a = 0.084±0.022
c	2	–	–	+	–	175	0.563	
d	1.73	–	0.27	+	–	175	0.610	d−c = 0.047±0.018
III a	2	–	–	–	–	200	0.578	
b	2	10^{-3}	–	–	–	200	0.738	b−a = 0.160±0.012
c	2	–	–	–	+	200	0.580	
d	2	10^{-3}	–	–	+	200	0.686	d−c = 0.106±0.012
IV a	2	–	–	–	–	185	0.662	
b	1.68	–	0.32	–	–	185	0.767	b−a = 0.105±0.011
c	2	–	–	–	+	185	0.647	
d	1.68	–	0.32	–	+	185	0.688	d−c = 0.041±0.037

TABLE IX

Approximate estimation of insulin concentration in serum

	Medium	Serum dil.	No. of observations	"Insulin" effect	Estimated insulin concentration (*U./ml. serum*)	Source
I	a) buffer + serum	1:5	6	$+0.182 \pm 0.072$	$\pm 5 \times 10^{-4}$	normal dog
	b) buffer + serum + 10^{-4} U./ml.		6	$+0.185 \pm 0.061$		
II	a) buffer + serum	4:25	6	$+0.175 \pm 0.049$	$< 6.25 \times 10^{-2}$ ($\pm 10^{-4}$?)	normal human
	b) buffer + 5×10^{-3} U./ml.		6	$+0.327 \pm 0.059$		
III	a) buffer + serum	4:25	6	$+0.187 \pm 0.035$	$\pm 6.25 \times 10^{-4}$	normal human
	b) buffer + 10^{-4} U./ml.		6	$+0.181 \pm 0.028$		
IV	a) buffer + serum	4:25	6	$+0.095 \pm 0.053$	$\pm 6.25 \times 10^{-5}$	human diabetic under insulin treatment
	b) buffer + 10^{-5} U./ml.		6	$+0.097 \pm 0.060$		
V	a) buffer + serum	4:25	6	$+0.222 \pm 0.077$	$> 6.25 \times 10^{-5}$ ($\pm 10^{-3}$?)	human diabetic after insulin injection
	b) buffer + 10^{-6} U./ml.		6	$+0.096 \pm 0.034$		

periments, we have taken care to include one or two control observations with pure insulin of known concentration with every serum determination. From the figures thus obtained, Table IX has been constructed. It summarizes the results of five experiments in which the insulin effects of different sera were compared with the effects of a known quantity of insulin which had been added to the glucose buffer solution in the control vessel. From these observations it will be seen that a normal dog serum contained about 5×10^{-4} units/ml. One normal human serum was found to contain about 10^{-4} units/ml., in any event less, in this particular experiment than 6.25×10^{-2} units/ml. The value obtained for the serum of a human diabetic under insulin treatment corresponded very well with the figures of the control experiment, which were 6.25×10^{-5} units of added insulin/ml. In the last experiment, in which the control vessel contained 10^{-5} units/ml., the serum produced a much higher glucose utilization than the control which, according to our standardization curve, would yield about 5×10^{-4} units/ml. This high value was obtained in a serum of a diabetic patient taken 15 minutes after an insulin injection of 40 units.

From these figures and several others, we tentatively suggest that the insulin concentration in normal serum is between 6.25×10^{-5} and 6.25×10^{-4} units/ml. In diabetic coma, the concentration is lower, being well below 10^{-5} units/ml. In cases of diabetes, while under insulin treatment, we have found a normal or slightly higher insulin content of the serum. The data from some of our experiments suggest that shortly after an injection of insulin, the insulin content of the serum may be higher than normal, but a sufficient number of observations have not yet been made to establish this as fact. On the other hand, we have repeatedly determined the insulin content in the serum of diabetic patients who, for some reason or other, had stopped taking insulin. One of these was a 78 year old woman who had discontinued her insulin injections after a stroke, and was admitted four days later with moderate diabetic acidosis, but not in coma. The insulin content of her blood was found to be 10^{-5} units/ml. It must be pointed out that the determination of the insulin content of the serum by the rat diaphragm method may not be sensitive enough to detect small differences in insulin content, but as it is, we have found a significant lowering of the insulin concentrations of the serum only in diabetic coma, not in patients with simple diabetes.

Hyperinsulinism and functional hypoglycaemia

The estimation of the insulin in serum offers possibilities for a direct demonstration of true hyperinsulinaemia and a differential diagnosis of this condition from "functional hypoglycaemia." We have tested two sera of cases of functional hypoglycaemia.[3] In both cases the insulin con-

[3] We are indebted to Dr. Lubsen and Dr. Meyer for these serum samples.

TABLE X

Estimation of insulin content of serum of patient with "functional" hypoglycaemia

Addition	Glucose effect (mg./100 mg. tissue)	Glycogen effect (mg./100 mg. tissue)
Insulin 10^{-5} U./ml.	-0.012 ± 0.090	$+0.066 \pm 0.024$
Pat. serum 1 : 6	-0.001 ± 0.051	$+0.122 \pm 0.018$
Insulin 10^{-3} U./ml.	$+0.217 \pm 0.067$	$+0.187 \pm 0.061$
Conclusion:	$\text{Ins.}_{\text{serum}} < 6 \times 10^{-3}$	$6 \times 10^{-5} < \text{Ins.}_{\text{serum}} < 6 \times 10^{-3}$

S = statistically significant difference.
$\not S$ = no statistically significant difference.

tent of the serum was found to be within normal limits. Table X summarizes the estimation of the insulin content in one of these cases. Quarter-diaphragms were used. One flask contained glucose-buffer solution, the second diluted serum. To the glucose-buffer solution in the third and fourth flasks, insulin was added in quantities of 10^{-5} and 10^{-3} units/ml., respectively. The diaphragms in this experiment were relatively insensitive, with the result that neither the addition of 10^{-5} units/ml. nor the addition of the patient's serum had a significant effect on glucose utilization. Judging from the glucose utilization, the insulin content of this patient's serum was significantly below 6.25×10^{-3} units of insulin, but was not significantly higher than 6.25×10^{-5} units/ml. A significant increase in glycogen synthesis was produced by the addition of 10^{-5} units of insulin/ml. The serum effect was significantly higher, but the effect of 10^{-3} units was

TABLE XI

Estimations of insulin content in serum of patient with hyperinsulinism

Exp.	Medium	Glucose effect (mg. glucose/100 mg. wet tissue)	Glycogen effect (mg. glucose/100 mg. wet tissue)
I	a) Patient's serum 4 : 25	0.219 ± 0.046	0.136 ± 0.061
	b) Normal serum 4 : 25	0.187 ± 0.035	0.086 ± 0.047
	c) Insulin 10^{-4} U./ml.	0.181 ± 0.028	0.072 ± 0.028
II	a) Patient's serum 4 : 25	0.218 ± 0.061	0.120 ± 0.070
	b) Redissolved lyophylized patient's serum 4 : 25	0.217 ± 0.028	0.123 ± 0.060
	c) Insulin 10^{-4} U./ml.	0.199 ± 0.051	0.098 ± 0.040

Conclusion: From experiment I: Insulin concentration in first serum sample $> 6.25 \times 10^{-4}$ U./ml. ($\pm 10^{-3}$ U./ml. ?).
From experiment II: Insulin concentration in second serum sample $\pm 6.25 \times 10^{-4}$ U./ml. Insulin content did not decrease by lyophylisation.
* S = Values differ significantly.
$\not S$ = No significant difference.

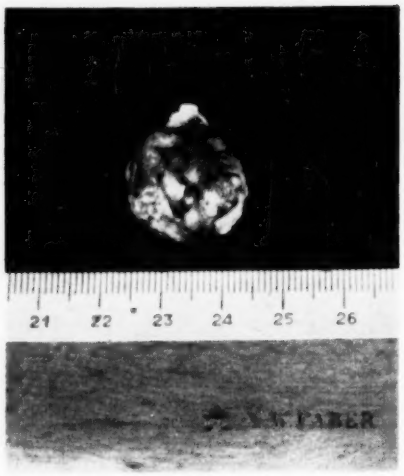

FIG. 6. PANCREATIC ISLET CELL ADENOMA, DIAGNOSED BY DIRECT ESTIMATION OF THE INSULIN CONTENT IN THE PATIENT'S SERUM

higher than that of the serum. From these results it appeared that the insulin content of the serum was lower than 6.25×10^{-3}, but higher than 6.25×10^{-5} units/ml. The conclusion that the insulin content in this case, as far as could be ascertained by our method, was about normal was supported later by the absence of an islet cell tumor at the operation and the success of a high protein, high fat diet.

We have examined only one case of hyperinsulinism thus far. The results of two determinations are given in Table XI. In both experiments the insulin content was found to be higher than that of normal serum, which was tested on the same diaphragms, and also significantly higher than the effect of 10^{-4} units of insulin. As the serum had been diluted 4 : 25, the insulin content of the serum, read from the concentration curves, was estimated at about 3×10^{-3} units/ml., which is almost five times as much as the highest level of normal. At operation an islet cell adenoma was discovered and extirpated [1] (Figure 6).

DISCUSSION

The quantitative aspects of the action of insulin on the isolated rat diaphragm have been

[1] We are indebted to Dr. Lips for sending us the serum of this case.

studied by Stadie and Zapp (5), Krahl and Park (6), and Willebrands and associates (9). The first named investigators found the lowest limits at which an effect could be detected to be 10^{-3} units/ml.; Krahl and Park found significant effects at 10^{-4} units ml., and Willebrands and coworkers, at 5×10^{-6} units ml. The difference between the results of these authors are best explained by the variations in the sensitivity of the diaphragms as illustrated in Figure 2. Tuerkischer and Wertheimer (7) and Perlmutter and Greep (12) have been, as far as we are aware, the only authors who have demonstrated a stimulating effect of human blood serum upon the glycogen synthesis of the isolated rat diaphragm. However, they have not followed up their observations and we suppose that the great variability in sensitivity of the diaphragms has discouraged them from using this relatively simple *in vitro* technique for the determination of the insulin concentration of serum.

Several attempts have been made, however, to determine the insulin concentration of serum by testing the drop in blood sugar after the injection of serum into animals which had been made especially sensitive to small amounts of insulin (13). For this purpose Gellhorn, Feldman, and Allen (14, 15) and Eston and Eston (16) have used hypophysectomized adreno-demedulated rats. Anderson and associates (17–19) and Bornstein (20, 21) introduced a further improvement when they found that the alloxan-diabetic hypophysectomized adreno-demedulated or adrenalectomized rat was an even better test object for the detection of minute amounts of insulin. The last named author found the insulin content of human plasma to be about 10^{-4} units ml. in the fasting state, rising to about 3×10^{-4} units ml. after oral administration of glucose. These figures agree very well with our finding that the insulin content of normal serum must be somewhere between 6.25×10^{-4} and 6.25×10^{-5} unit ml., although our technique does not enable us to distinguish significantly between concentrations of 10^{-4} and 3×10^{-4} units ml. For this reason, we are also unable to pass a judgment on the recent claim of Bornstein and Lawrence (22) that there exist two types of human diabetes, one with and the other without insulin in the serum.

SUMMARY

The results of a quantitative study of the effect of insulin on the glucose utilization of the isolated rat diaphragm are reported. Although the sensitivity of the isolated rat diaphragm to small amounts of insulin is variable, it can be demonstrated by this method that normal human and dog sera contain a substance which has an insulin-like action on glucose utilization and glycogen synthesis of the isolated rat diaphragm. In sera of patients with diabetic coma, and of dogs after complete pancreatectomy, the insulin-like activity was either absent or considerably diminished.

The increase in the glucose utilization and glycogen synthesis upon the addition of serum may be used as a means of estimating the insulin content of blood. Normal blood serum contains between 6.25×10^{-3} and 6.25×10^{-4} units of insulin per ml. Using this method, accurate diagnoses of "functional" hypoglycaemia (two cases) and of islet cell adenoma (one case) were made. The possibilities and limitations of the method are discussed.

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REFERENCES

1. Takane, R., Uber Atmung und Kohlehydratumsatz tierischer Gewebe. II. Mitteilung: Atmung und Kohlehydratumsatz in Leber und Muskel des Warmbluters. Biochem. Ztschr., 1926, **171**, 403.
2. Gemmill, C. L., The effect of insulin on the glycogen content of isolated muscles. Bull. Johns Hopkins Hosp., 1940, **66**, 232.
3a. Gemmill, C. L., and Hamman, L., Jr., The effect of insulin on glycogen deposition and on glucose utilization by isolated muscles. Bull. Johns Hopkins Hosp., 1941, **68**, 50.
3b. Gemmill, C. L., The effects of glucose and of insulin on the metabolism of the isolated diaphragm of the rat. *Ibid.*, 329.
4. Villee, C. A., and Hastings, A. B., The metabolism of C^{13}-labelled glucose by the rat diaphragm *in vitro*. J. Biol. Chem., 1949, **179**, 673.
5. Stadie, W. C., and Zapp, J. A., Jr., The effect of insulin upon the synthesis of glycogen by rat diaphragm *in vitro*. J. Biol. Chem., 1947, **170**, 55.
6. Krahl, M. E., and Park, C. R., The uptake of glucose by the isolated diaphragm of normal and hypophysectomized rats. J. Biol. Chem., 1948, **174**, 939.

7. Tuerkischer, E., and Wertheimer, E., The *in vitro* synthesis of glycogen in the diaphragms of normal and alloxan-diabetic rats. Biochem. Ztschr., 1948, **42**, 603.

8. Gilmore, R. C., Jr., and Samuels, L. T., The effect of previous diet on the metabolic activity of the isolated rat diaphragm. J. Biol. Chem., 1949, **181**, 813.

9. Willebrands, A. F., Groen, J., Kamminga, C. E., and Blickman, J. R., Quantitative aspects of the action of insulin on the glucose and potassium metabolism of the isolated rat diaphragm. Science, 1950, **112**, 277.

10. Gey, G. O., and Gey, M. K., The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. Am. J. Cancer, 1936, **27**, 45.

11. Somogyi, M., Determination of blood sugar. J. Biol. Chem., 1945, **160**, 69.

12. Perlmutter, M., and Greep, R. O., Effect of insulin upon the *in vitro* glucose utilization and glycogenesis of the diaphragm of normal and pituitarectomized rats. Adaptation of this technique as an assay for serum content of insulin and anti-insulin substances. Federation Proc., 1948, **7**, 92.

13. Waters, E. T., and Best, C. H., The importance of developing an accurate method for the estimation of insulin in the blood. Proc. Am. Diabetes A., 1947, **7**, 169.

14. Gellhorn, E., Feldman, J., and Allen, A., Assay of insulin on hypophysectomized, adreno-demedulated, and hypophysectomized-adreno-demedulated rats. Endocrinology, 1941, **29**, 137.

15. Gellhorn, E., Feldman, J., and Allen, A., Insulin concentration in the blood of normal and pancreatectomized dogs. Endocrinology, 1941, **29**, 849.

16. Cited by Waters and Best (13), p. 174.

17. Anderson, E., Lindner, E., and Sutton, V., A sensitive method for the assay of insulin in blood. Am. J. Physiol., 1947, **149**, 350.

18. Anderson, E., and Long, J. A., The effect of hyperglycemia on insulin secretion as determined with the isolated rat pancreas in a perfusion apparatus. Endocrinology, 1947, **40**, 92.

19. Anderson, E., and Long, J. A., The hormonal influences on the secretion of insulin, *in* Recent Progress in Hormone Research, edited by Pincus, G. Academic Press, New York, 1948, Vol. II, p. 209.

20. Bornstein, J., A technique for the assay of small quantities of insulin using alloxan diabetic, hypophysectomized, adrenalectomized rats. Austr. J. Exper. Biol. & M. Sc., 1950, **28**, 87.

21. Bornstein, J., Normal insulin concentration in man. Austr. J. Exper. Biol. & M. Sc., 1950, **28**, 93.

22. Bornstein, J., and Lawrence, R. D., Two types of diabetes mellitus, with and without available plasma insulin. Brit. M. J., 1951, **1**, 732.

THE EFFECT OF SERUM FROM INSULIN-RESISTANT CASES ON THE COMBINATION OF INSULIN WITH THE RAT DIAPHRAGM [1]

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Insulin resistance is currently believed to be associated with insulin-neutralizing substances in the blood. Lowell (1), employing the mouse convulsion assay for insulin, has shown that the injection of mixtures of insulin and serum from insulin-resistant diabetic patients into mice caused a significantly smaller incidence of convulsive symptoms than the injection of the same amounts of insulin alone. He points out, however, that the quantitative interpretation of these experiments is difficult because of the variations of the response of the animals to insulin and the non-specific effects of the injection of serum. It would be desirable, therefore, to employ a method for the determination of insulin-inactivating properties of serum which does not involve the administration of insulin-serum mixtures to the intact animal. In the course of our recent work on the chemical combination of insulin with the rat diaphragm (*2*, 3) it became apparent that this technique would provide a method for the detection of insulin-inactivating substances in any solution. Accordingly, we have tested serum from normal, diabetic, and insulin-resistant subjects and found, in confirmation of the results of Lowell, that serum from insulin-resistant patients has the property of opposing the action of insulin.

METHODS

In our procedure (*2*), the synthesis of glycogen from glucose by paired rat hemi-diaphragms is measured. Insulin is added to the appropriate dilution of serum at 25° C., and after five minutes one of the hemi-diaphragms is placed in this solution for an additional one minute. The paired control hemi-diaphragm is placed for one minute in serum to which no insulin has been added. Both hemi-diaphragms are then washed twice for 30 seconds each in 25 ml. phosphate-saline medium and transferred to vessels containing medium of the following composition: 0.087 M NaCl, 0.005 M $MgCl_2$, 0.040 M sodium phosphate, and 0.4% glucose, pH 6.8. The vessels are gassed with oxygen and shaken in a water bath at 38° C. for 90 minutes. The difference in the extent of glycogen synthesis during the period of equilibration is a measure of the insulin effect. Since the amount of insulin combining with the diaphragm as measured by the effect on glycogen synthesis has been shown to vary in a regular manner with the insulin concentration during the one minute exposure (3), it is evident that a change in the effective concentration of insulin brought about by the addition of serum should be measurable by this method. It should be pointed out, however, that the method suffers from severe limitations caused by the biological variability of the response of the diaphragm to insulin and to errors necessarily introduced by the many manipulations involved. For each assay several experiments are carried out and the mean insulin effect recorded. We believe that decreases in insulin concentration of 50% or more should be clearly demonstrable by this method.

RESULTS

It may be seen from Table I that at an insulin concentration of 0.1 unit per ml., the insulin effect is lower when the diaphragm is exposed to an insulin-serum mixture than when serum is omitted. However, sera from normal persons and non-resistant diabetic patients do not differ significantly in their ability to lower the effective concentration of insulin.

In three cases of insulin resistance, requiring 300 to 800 units per day, the insulin effect after incubation with serum of dilution 1:1 was not significantly different from zero (cases I, II, and III, Table I). Where the insulin resistance was mild as in cases IVa, V, and VI, the insulin effects were not significantly lower than in the presence of normal serum.[2]

[1] The work reported in this paper was supported in part by grants from the National Institutes of Health, United States Public Health Service, and the Insulin Grants Committee of the Lilly Research Laboratories.

[2] We should like to express our thanks to the physicians who kindly made available the sera used in these experiments. Case I was a patient of Dr. Norman Schnee-

TABLE I

The effect of sera from normal and insulin-resistant individuals on the combination of insulin with rat diaphragm

The serum was diluted with phosphate-saline medium as indicated below and incubated for five minutes at room temperature with 0.1 unit of insulin per ml. A normal rat hemi-diaphragm was added for one minute, washed and allowed to synthesize glycogen for 90 minutes. The control hemi-diaphragm was treated in the same manner, except for the omission of insulin. The insulin effect is the difference in final glycogen content of the two hemi-diaphragms.

Source of serum	Insulin requirement	Dilution	No. of expts.	Insulin effect on glycogen synthesis, *micromoles (glucose equiv.) per gram* Mean ±SEM
	(units/day)			
No serum present	—		75	8.3 ±0.52
Normal persons	0	1 : 1	11	5.6 ±0.92
Mild diabetics	0–20	1 : 1	6	5.3 ±1.05
Insulin-resistant, severe				
Case I	800	1 : 1	5	0.6 ±0.67
		1 : 3	5	2.1 ±1.26
Case II	600	1 : 1	5	0.5 ±0.98
		1 : 10	6	5.0 ±1.41
Case III (a)	300	1 : 1	5	2.2 ±0.98
		1 : 3	5	4.7 ±1.15
(b)	170	1 : 1	4	4.0 ±1.75
Insulin-resistant, mild				
Case IV (a)	200	1 : 1	5	3.2 ±1.09
(b)	20	1 : 1	4	7.0 ±1.46
Case V	150	1 : 1	4	4.8 ±0.71
Case VI	150	1 : 1	3	5.1 ±2.25

The samples of serum from the more severe cases of insulin resistance were studied in different dilutions as indicated in Table I. It is evident that the more dilute solutions are less effective in lowering the concentration of insulin.

Samples of serum from cases III and IV were obtained and studied at different times during the observation of the patients. The insulin requirements of case III were of the same order of magnitude when the two samples were taken and there were no significant differences between the activity of the two samples. In case IV, however, the second sample was taken at a time when no insulin resistance was observed and the insulin effect was significantly higher than with the first sample.

berg of Mount Sinai Hospital, Philadelphia, Pa. Serum from case II was obtained from Dr. Leon S. Smelo of the Medical Diagnostic Clinic, Birmingham, Ala. Serum from case III was obtained from Dr. W. Wallace Dyer of the Hospital of the University of Pennsylvania, Philadelphia, Pa. The sera from the patients with mild insulin resistance were from the Hospital of the University of Pennsylvania. Case III has been reported by Spoont and Dyer (4).

Table II shows the results of a more extensive study carried out with serum from a patient of Dr. John Howard of the Johns Hopkins Hospital.[3] During the course of this man's illness he required as much as 6,000 units of insulin per day to control his diabetes. All of the samples of serum studied except the last which was taken after recovery of the patient possesed insulin-inactivating properties at a dilution of 1 : 4. The first sample taken at the height of the resistance to insulin was active at a dilution of 1 : 4 and 1 : 10. Even at a dilution of 1 : 100 there appeared to be some activity present since the mean insulin effect was significantly lower than in the absence of serum (Table I). The patient was treated with ACTH because of the allergic phenomena present and in the course of a week the resistance was improved, with a daily insulin requirement of less than 500 units. Serum drawn at this time still showed insulin-inactivating ability at a dilution of 1 : 4.

Except for the demonstration that the last sample of serum taken at a time when the patient had recovered completely from the insulin resistance did not possess any insulin-inactivating properties, we have not been able to observe any consistant correlation between the activity of the serum and the clinical course of the patient. In

[3] We should like to express our appreciation to Drs. John E. Howard and Ernest Brown of Johns Hopkins Hospital for sending us these samples of serum from this unusual case of insulin resistance.

TABLE II

The effect of serum from a severe case of insulin resistance on the combination of insulin with rat diaphragm
(Procedure was the same as that indicated in Table I)

Serum no.	Date	Insulin requirement	Dilution of serum	No. of expts.	Insulin effect, *micromoles (glucose equiv.) per gram* Mean ±SEM
		(units/day)			
1	7/22/50	3,000–6,000	1:4	4	2.0±0.50
			1:10	9	1.7±1.36
			1:100	10	4.4±1.41
2	9/15/50	1,500	1:4	4	2.6±0.74
3	9/28/50	600*	1:4	5	1.9±1.42
4	10/ 2/50	100*	1:4	4	0.1±2.80
5	10/ 7/50	0*	1:4	4	2.2±0.50
6	10/20/50	0*	1:4	5	1.7±1.53
			1:10	7	2.8±2.27
			1:100	5	5.6±1.82
7	1/ 9/51	0–20	1:4	4	6.9±0.83

* Patient was receiving ACTH therapy.

the dilutions of 1:4 the samples of the serum all showed the presence of insulin-inactivating substances. A better quantitative comparison might have been achieved by determining the highest dilution at which each serum still exhibited insulin-inactivating properties. Such a study, however, would require a very large number of experiments and would at best be only semi-quantitative due to the limitations of the assay method.

It is interesting that samples 5 and 6 still exhibited insulin-inactivating activity although the insulin resistance at that time had been overcome. The possibility suggests itself that the activity at this stage might be a reflection of the ACTH therapy.

A detailed discussion of the illness of this patient and of the results of the treatment with ACTH has been published by Howard (5).

A number of experiments were carried out in order to obtain some information about the nature of the factor in serum responsible for the insulin-inactivating property. It was found that the inactivating property of serum at a given dilution could be overcome by raising the concentration of insulin. A mixture of sera from samples 2 and 3 (Table II) in a dilution of 1:4 was used. Both these samples have been shown to inactivate 0.1 unit of insulin per ml. at this dilution. It was found that when the concentration of insulin was raised to 1 unit per ml. an insulin effect of 12.6 ± 4.12 micromoles per gram was obtained ($N = 4$). This is not significantly different from the insulin effect at this concentration of insulin in the absence of serum. It is apparent from this experiment and from the experiments with different dilutions of serum and constant insulin concentration that the extent of the insulin effect is a function of the relative amounts of serum and insulin.

We have studied whether treatment of the diaphragm with serum from an insulin-resistant patient will make it refractory to the subsequent action of insulin. Sample 1 (Table II) was used for the experiment. This sample of serum completely abolished the insulin effect in a dilution of 1:10 and even appeared to have some activity at a dilution 1:100. One hemi-diaphragm was equilibrated in this serum at a 1:4 dilution for five minutes at 38° C. The control hemi-diaphragm was equilibrated in medium for the same length of time. Both diaphragms were then immersed in an insulin solution at a concentration of 0.1 unit per ml. for one minute, washed and allowed to synthesize glycogen for 90 minutes at 38° C. In five experiments there was no significant difference between the final glycogen contents of the paired hemi-diaphragms indicating that the treatment of the diaphragm with serum had not influenced its ability to synthesize glycogen or respond to insulin.

It does not appear that hyperglycemia by itself is a factor in the production of insulin-inactivating substances. No insulin-inactivating properties could be demonstrated in a sample of serum from a patient with a brain lesion resulting in excessive hyperglycemia (1,500 mg.%). It was also noted that the blood sugar concentrations in the samples of serum from the insulin-resistant cases studied varied considerably and did not bear any relation to the ability of the serum to inactivate insulin. The presence of glucose during the exposure to insulin has been shown in previous experiments to have no effect on the combination of insulin with the diaphragm (2).

DISCUSSION

The experiments reported here indicate that the technique involved in studying the combination of insulin with the isolated rat diaphragm provides a method for the estimation of insulin-neutralizing substances in serum or other solutions. By this means, we have shown that sera from normal and diabetic human subjects contain significant amounts of such substances and that the serum of insulin-resistant individuals contains excessive amounts. Presumably, these substances combine with or inactivate some or all of the added insulin during the five minute incubation period before the diaphragm is exposed to the insulin-serum mixture.

An alternative explanation of these experimental results is that some substance in the serum from insulin-resistant individuals is capable of combining with the diaphragm during the one minute incubation period rendering it less susceptible to the action of insulin. Such an effect has been obtained in experiments with pitui-

tary extract (6). However, our negative results in an attempt to demonstrate an action of serum itself on the diaphragm lead us to believe that substances present in the serum combine with or inactivate insulin in the serum-insulin mixture. The experiments of Lowell (1) are also best interpreted in this light.

The origin and chemical nature of the active principle demonstrated in serum from insulin-resistant individuals remain a matter for speculation. It is possible that a reaction of the nature of an antigen-antibody combination takes place. If such is the case, it should be possible to demonstrate a quantitative relationship between the ratio of serum to insulin in the serum-insulin mixture used and the ability to prevent the action of insulin on the diaphragm.

It would also be of great importance to determine whether the effects obtained with serum are dependent on the type of insulin used, particularly whether the insulin-inactivating properties of serum are found when human insulin is used or whether it is only demonstrable with insulin obtained from animal sources.

It is of interest here to note that Brown (7) has been able to demonstrate that serum from the insulin-resistant patient of Dr. Howard had the property in high dilutions of causing the agglutination of insulin-coated polystyrene particles. This phenomenon was observed to a greater or smaller extent with all the samples of serum in Table II except the last. These results are, therefore, in agreement with our findings since only with the last sample of serum did we fail to demonstrate the ability to lower the effect of insulin on the diaphragm.

The ability of normal human serum to lower the effective concentration of insulin is interesting and leads one to speculate whether insulin may not normally be present in the blood in a form combined with some serum component.

SUMMARY

The ability of human serum to influence the combination of insulin with the isolated rat diaphragm has been studied. The combination of insulin with the diaphragm, as measured by its effect on glycogen synthesis, was found to be significantly decreased in the presence of serum from normal or diabetic individuals. We have concluded from this that serum contains substances which combine with or inactivate insulin. Serum from insulin-resistant patients appears to contain greatly increased amounts of such substances.

Experiments on the combination of insulin with tissue offer interesting possibilities in the study of the phenomenon of insulin resistance.

REFERENCES

1. Lowell, F. C., Immunologic studies in insulin resistance. II. The presence of a neutralizing factor in the blood exhibiting some characteristics of an antibody. J. Clin. Invest., 1944, **23**, 233.
2. Stadie, W. C., Haugaard, N., Marsh, J. B., and Hills, A. G., The chemical combination of insulin with muscle (diaphragm) of normal rat. Am. J. M. Sc., 1949, **218**, 265.
3. Stadie, W. C., Haugaard, N., and Marsh, J. B., Factors influencing the combination of insulin with muscle from normal rats. J. Biol. Chem., 1951, 189, 53.
4. Spoont, S., and Dyer, W. W., Insulin resistance associated with local and general allergy to insulin. J.A.M.A., 1951, **145**, 558.
5. Howard, J. E., *in* discussion on Margen, S., Michaels, G. D., Boling, L. A., and Kinsell, L. W., Hormonal regulation of fat metabolism. II. Effects of ACTH and certain steroid hormones upon the utilization of infused aceto-acetate and octonoic acid, *in* Proceedings of the Second Clinical ACTH Conference, edited by Mote, J. R. The Blakiston Co., New York, 1951, Vol. I., Chap. 32, p. 318.
6. Stadie, W. C., Haugaard, N., Hills, A. G., and Marsh, J. B., Hormonal influences on the chemical combination of insulin with rat muscle (diaphragm). Am. J. M. Sc., 1949, **218**, 275.
7. Brown, E. C., Jr., Personal communication.

THE EFFECT OF INTRAVENOUS PAPAVERINE HYDROCHLORIDE ON THE CEREBRAL CIRCULATION [1, 2]

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Papaverine, one of the benzylisoquinoline derivatives of opium, has found its primary usefulness as a relaxer of the smooth muscle of blood vessels (1). Numerous reports are to be found in the literature as to its effectiveness in the treatment of occlusive and spastic conditions of the pulmonary (2), coronary (3) and peripheral arteries (4–6). In recent years, Russek and Zohman have reported clinical studies in which they felt papaverine had been demonstrated to be an effective therapeutic agent in the treatment of vasospastic conditions involving the cerebral vessels (7). Experimental demonstration of the effect of papaverine hydrochloride on the cerebral vessels has been limited to a report by Shenkin of the effect of 0.06 gm. of the drug intravenously in four patients judged to have decreased cerebral circulation (8). He noted no significant change in cerebral circulatory functions other than a reduction of mean arterial blood pressure and cerebral vascular resistance. The purpose of this paper is to report our findings on the effects of intravenous papaverine on cerebral blood flow and metabolism in 18 subjects, as measured by the nitrous oxide technique (9).

METHOD

Eighteen hospital patients were chosen, the two largest component groups being patients with acute and chronic liver disease (seven), and normal middle-aged and elderly people (five). There was no purpose in this selection of patients except that the cerebral metabolism of these groups was under study for other purposes. The remainder of the subjects (six) were chosen at random. All patients were males and ranged in age from 25 to 79 years. A control cerebral blood flow was performed using the modification of the original nitrous oxide technique previously described (10), in which continuous simultaneous samples of blood are drawn from the internal jugular bulb and femoral artery over the ten-minute period of nitrous oxide inhalation to measure mean arterio-venous nitrous oxide difference. The papaverine was administered intravenously in doses of 0.2 gm. in 200 cc. normal saline over a period of about 15 to 20 minutes. The second blood flow measurement was begun approximately five to eight minutes after the intravenous solution had been started. Arterial pressure measurements were made by the auscultatory method every two minutes throughout the procedures, with the arm held at heart level. Mean pressures were calculated from the formula MP = Diastolic pressure + ⅓ pulse pressure. These readings were checked on several patients by direct pressure readings on a U tube mercury manometer. In all instances calculated and direct pressures agreed closely. Blood samples for oxygen determinations were drawn before and after each blood flow measurement, and the average was used as the final arterial-cerebral venous oxygen difference. The oxygen measurements were done by the spectrophotometric method of Hickam and Frayser (11). Cerebral oxygen consumption and cerebral vascular resistance were calculated in the usual manner. Cerebral venous oxygen tension was not measured directly, but was computed from cerebral venous oxygen content by means of an oxygen dissociation curve drawn for pH 7.4.

RESULTS

The data obtained from these studies are presented in Table I. As may be seen, there was a statistically significant increase in cerebral blood flow (CBF) from a mean of 56.6 to 63.9 ml./100 gm. brain/min. ($p = 0.013$), an increase of 13%. This was associated with a statistically significant decrease in cerebral vascular resistance (CVR), from a mean of 1.67 to 1.40 mm. Hg/ml. blood/100 gm. brain/min. ($p = < .01$). The mean

[1] This investigation was supported in part by a research grant from the National Heart Institute, of the National Institutes of Health, Public Health Service.

[2] Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

arterial-cerebral venous oxygen difference decreased from 6.64 to 5.86 vol.% ($p = < .01$), commensurate with the increase in CBF, so that cerebral oxygen consumption showed no significant change. Mean arterial pressure fell from 98 to 85 mm. Hg, a statistically significant decrease. Mean cerebral venous oxygen tension increased slightly but significantly ($p < 0.01$), from 30.5 to 32.1. As can be seen from Figure 1, cerebral blood flow actually decreased slightly in five of the 18 subjects. The reasons for this cannot be stated with surety, but it seems likely that it was due to experimental error in the procedure, perhaps in the analysis of the blood samples for nitrous oxide, because in four of these five patients, the arterial-cerebral venous oxygen difference decreased, and in the fifth it was unchanged. Since the determination of the arteriovenous oxygen difference is subject to less error than measurement of the cerebral blood flow, it is probable that the blood flow determinations were inaccurate. In only one of these five (R. W., Table I) was the difference between the control and papaverine determination outside experimental error. The subjects in whom this phenomenon occurred had no physiological similarity

TABLE I

Comparison of cerebral functions (controls and after i.v. papaverine)

Pt.	Age	Diagnosis	Cerebral blood flow (*ml./min./100 gm. brain*)		Arterial-cerebral venous O_2 difference (vol. %)		Cerebral O_2 consumption (*ml. O_2/min./100 gm. brain*)		Mean arterial pressure (*mm. Hg*)		Cerebral vascular resistance (*units*)		Cerebral venous O_2 tension (*mm. Hg*)	
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
A. G.	34	Homologous serum jaundice	53	74	5.99	5.56	3.15	4.12	81	76	1.54	1.03	27	28
D. H.	42	Arthritis, infectious	51	60	7.73	7.03	3.93	4.21	85	84	1.67	1.40	26	29
M. R.	79	Normal old age	40	53	9.32	7.27	3.73	3.85	94	95	2.35	1.79	25	29
C. B.	40	Laennec's cirrhosis	63	71	5.80	4.73	3.65	3.36	101	85	1.60	1.20	31	33
H. M.	38	Laennec's cirrhosis	45	44	4.78	3.30	2.15	1.45	87	80	1.93	1.82	38	44
P. M.	58	Laennec's cirrhosis	64	68	4.90	4.70	3.13	3.19	90	85	1.41	1.25	25	26
C. B.	58	Normal middle age	47	74	6.54	5.21	3.08	3.86	90	68	1.91	0.92	31	36
R. L.	33	Viral hepatitis	55	59	7.76	6.96	4.27	4.11	93	80	1.69	1.29	29	32
M. S.	50	Normal middle age	66	79	6.66	5.66	4.40	4.47	97	91	1.47	1.15	32	34
G. O.	56	Laennec's cirrhosis	55	54	5.12	4.13	2.81	2.23	81	72	1.47	1.33	34	38
C. C.	53	Normal middle age	59	67	7.13	6.54	4.21	4.37	79	78	1.34	1.16	30	32
R. E.	51	Cerebral vascular disease	47	45	6.98	5.55	3.28	2.50	92	98	1.96	2.16	30	34
J. R.	44	Normal middle age	60	64	6.15	5.58	3.69	3.57	104	88	1.74	1.37	32	33
M. D.	31	Idiopathic epilepsy	63	83	6.31	6.18	3.95	5.13	102	97	1.62	1.17	33	33
R. G.	47	Multiple sclerosis	59	54	7.64	7.16	4.50	3.85	85	79	1.44	1.46	30	30
R. W.	34	Essential hypertension	65	46	7.46	7.30	4.85	3.36	145	132	2.23	2.87	32	33
P. B.	25	Viral hepatitis	51	73	8.36	7.57	4.27	5.53	83	70	1.63	0.96	29	30
L. R.	53	Mild bronchial asthma	75	82	4.94	4.81	3.70	3.94	79	78	1.05	0.95	36	35
		Mean values	56.6	63.9	6.64	5.86	3.72	3.74	98	85	1.67	1.40	30.5	32.1
		P values	0.013		<0.01		>0.5		<0.01		<0.01		<0.01	

$$t = \frac{\text{Mean difference}}{\text{Std. error of mean difference}}.$$

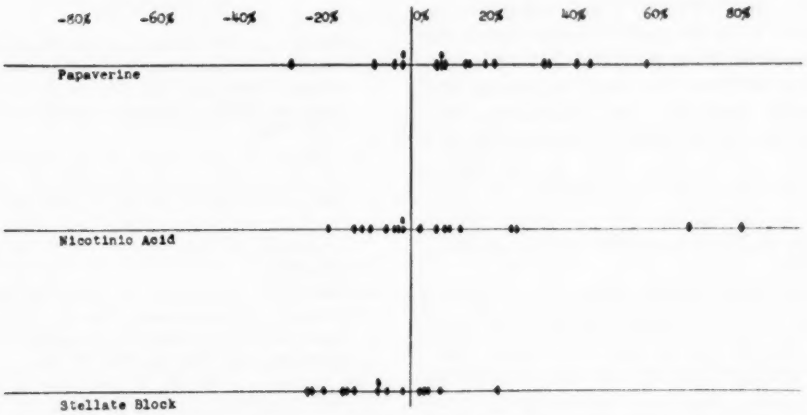

FIG. 1. INDIVIDUAL DETERMINATIONS OF CEREBRAL BLOOD FLOW, EXPRESSED AS PERCENTAGE CHANGE FROM THE CONTROL VALUES RESULTING FROM THE ADMINISTRATION OF PAPAVERINE, NICOTINIC ACID AND PROCAINE BLOCK OF THE STELLATE GANGLION

to explain it. Figure 1 also compares the percentage change in cerebral blood flow due to papaverine as compared to that due to nicotinic acid and stellate block, which were previously reported. The great preponderance of increases in blood flow following papaverine, as compared to the other two, is evident. The mean cerebral blood flow in the control series is somewhat below the value established for normal young men (mean of 65) (10) and is probably due to the fact that this study included a heterogeneous population of various disease states, as well as normal elderly subjects, for whom no normal mean value has been established.

DISCUSSION

In light of our past knowledge of papaverine, its action in causing relaxation of the smooth muscle of arteries and arterioles seems the best explanation for the decreased cerebral vascular resistance and increased cerebral blood flow noted is this study. There was no reason to suspect anoxia or decreased blood pH (12) of playing a role in these results for arterial O_2 content was unchanged after papaverine, as was blood pH in the several patients in whom it was measured. Although CO_2 tension of the blood was not measured, there was no reason to suspect that it was increased as a result of papaverine administration. The decrease in arterial pressure is suggestive evidence that the papaverine probably causes arteriolar dilatation, although this is certainly not localized to the brain. The possibility of contamination of internal jugular blood by extracerebral blood from increased blood flow to the skin of the face, thus causing abnormally high values for cerebral blood flow, must be considered just as with the intravenous administration of nicotinic acid (13). In none of the patients reported, however, was any facial flush noted during the administration of the papaverine. In six of the patients in whom cerebral blood flow was increased, the cerebral arteriovenous oxygen difference was measured after 100 mgm. nicotinic acid were given intravenously. In only one subject did the results indicate the presence of facial contamination, and these data were therefore discarded.

It is worthy of note that much of the repute gained by papaverine as a vasodilator is based on clinical rather than experimental studies. In coronary artery disease it is reported as being highly effective in relieving angina (3). In occlusive diseases of the peripheral arteries, many authors have reported papaverine as being a highly effective agent (5, 6), especially in relieving the vasospasm associated with acute obstructive episodes.

A recent study in normal subjects wherein the effect of various drugs on the peripheral vessels was recorded by use of venous occlusion plethys-

mography, skin temperatures and radioactive sodium uptake from the gastrocnemius muscle has shown that intravenous papaverine has a moderate vasodilating effect on the vessels in the skin and subcutaneous tissue in some individuals (14). The drug was not as effective as priscoline as a vasodilator and less than 50% of the subjects showed any significant vasodilatation. It is understood by the authors that laboratory studies of this sort may have no specific bearing on purely clinical data.

In recent years clinical studies have reported papaverine to be an effective drug in the treatment of diseases of the cerebral vessels. Russek and Zohman reported 46 cases of vascular encephalopathy due to hypertension, acute glomerulonephritis and similar disease states, all of whom showed definite beneficial effects from the administration of papaverine (7). It does not seem that the small increase in cerebral blood flow produced by papaverine would result in the profound clinical changes reported by these authors. On the other hand it should be pointed out that in none of our subjects was there reason to suspect acute cerebral vascular changes which might have been reversed by a cerebral vasodilating agent. For that reason final conclusions on the clinical efficacy of papaverine as a vasodilator must be reserved until such time as appropriate subjects can be studied.

These studies also indicate that just as in other portions of the vascular tree, a pronounced individual variability of response to papaverine is seen. It is of interest that papaverine does increase cerebral blood flow, since it is one of the few agents of those thus far reported that does so.

SUMMARY

1. Intravenous papaverine hydrochloride, in doses of 0.2 gm. administered in 20 minutes, results in a 13% increase in cerebral blood flow, a commensurate decrease in arterial-cerebral venous oxygen difference, with no change in cerebral oxygen consumption, and a decrease in cerebral vascular resistance and cerebral venous oxygen tension.

2. It is thought that the mechanism whereby papaverine increases cerebral blood flow and decreases cerebral vascular resistance, is its direct vasodilating effect on cerebral vessels.

REFERENCES

1. Goodman, L., and Gilman, A., The Pharmacological Basis of Therapeutics; A Textbook of Pharmacology, Toxicology and Therapeutics for Physicians and Medical Students. Macmillan Co., New York, 1941.
2. de Takats, G., and Jesser, J. H., Pulmonary embolism, suggestions for its diagnosis, prevention and management. J. A. M. A., 1940, **114**, 1415.
3. Elek, S. R., and Katz, L. N., Some clinical uses of papaverine in heart disease. J. A. M. A., 1942, **120**, 434.
4. Littauer, D., and Wright, I. S., Papaverine hydrochloride. Its questionable value as a vasodilating agent for use in treatment of peripheral vascular diseases. Am. Heart J., 1939, **17**, 325.
5. de Takats, G., The use of papaverine in acute arterial occlusion. J. A. M. A., 1936, **106**, 1003.
6. Allen, E. V., and MacLean, A. R., Treatment of sudden arterial occlusion with papaverine hydrochloride: report of case. Proc. Staff Meet., Mayo Clin., 1935, **10**, 216.
7. Russek, H. I., and Zohman, B. L., Papaverine in cerebral angiospasm (vascular encephalopathy). J. A. M. A., 1948, **136**, 930.
8. Shenkin, H. A., Effects of various drugs upon cerebral circulation and metabolism of man. J. Applied Physiol., 1951, **3**, 465.
9. Kety, S. S., and Schmidt, C. F., The nitrous oxide method for the quantitative determination of cerebral blood flow in man: theory, procedure and normal values. J. Clin. Invest., 1948, **27**, 476.
10. Scheinberg, P., and Stead, E. A., Jr., The cerebral blood flow in male subjects as measured by the nitrous oxide technique. Normal values for blood flow, oxygen utilization, glucose utilization, and peripheral resistance, with observations on the effect of tilting and anxiety. J. Clin. Invest., 1949, **28**, 1163.
11. Hickam, J. B., and Frayser, R., Spectrophotometric determination of blood oxygen. J. Biol. Chem., 1949, **180**, 457.
12. Kety, S. S., and Schmidt, C. F., The effects of altered arterial tensions of carbon dioxide and oxygen on cerebral blood flow and cerebral oxygen consumption of normal young men. J. Clin. Invest., 1948, **27**, 484.
13. Scheinberg, P., The effect of nicotinic acid on the cerebral circulation with observations of extracerebral contamination of cerebral venous blood in the nitrous oxide procedure for cerebral blood flow. Circulation, 1950, **1**, 1148.
14. Murphy, R. A., Jr., McClure, J. N., Jr., Cooper, F. W., Jr., and Crawley, L. G., The effect of priscoline, papaverine and nicotinic acid on blood flow in the lower extremity of man; comparative study. Surgery, 1950, **27**, 655.

METABOLISM OF INTRAVENOUS FRUCTOSE AND GLUCOSE IN NORMAL AND DIABETIC SUBJECTS [1, 2]

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Although fructose metabolism has been studied extensively in various animal species, no careful investigation has been made of the utilization and metabolic effects of intravenously administered fructose in humans with diabetes mellitus. It has long been recognized from animal studies that the metabolism of fructose differed significantly from that of glucose, despite the close similarity in chemical structure. This led to a trial of its utilization *orally* in patients with diabetes (1, 2). At first the fructose seemed to be well-utilized, but glycosuria increased gradually until the fructose balance became similar to that obtained with comparable amounts of glucose. In 1946 Root, Stotz and Carpenter (3) found that 50 gm. of fructose given orally to diabetic subjects produced greater rises in RQ, blood pyruvate and lactate than the same amount of glucose. Root concluded that "the intermediary metabolism of fructose is, with diabetic patients, as with normal subjects, different from that of glucose." Oral administration, however, cannot be expected to demonstrate quantitatively the effects of fructose, since both intestinal mucosa and liver would convert at least a portion to glucose (4, 5) as a consequence of the presence of both hexose isomerase and phosphatase. In the one mild diabetic patient in the series of Weichselbaum, Elman and Lund (6) where the tolerance to intravenous fructose was studied, the blood levels and urinary excretion of fructose seemed to be in the same range as those found in non-diabetic subjects.

Bueding, Wortis and Fein (7) showed that in the diabetic subject, in the absence of insulin, the administration of glucose orally was not accompanied by the same rise in blood pyruvate found in the normal subject under identical experimental conditions. With the injection of insulin in the diabetic subject a rise in pyruvate occurred. This seemed to indicate the possibility that insulin might act on one or more of the reactions in the Embden-Meyerhof scheme between glucose and pyruvate. In 1945 Price, Cori and Colowick (8) demonstrated *in vitro* that insulin diminished or abolished the inhibitory action of the anterior pituitary hormone on the enzyme, hexokinase, which catalyzes the phosphorylation of glucose. Some doubt has been raised as to the physiological significance of this action in view of the increased sensitivity to insulin of the hypophysectomized animal and the patient with destruction or degeneration of the pituitary gland (Simmond's disease). The need for further work concerning the nature of the metabolic defect in human diabetes is still apparent. Study of the intermediary metabolism of fructose, entering the Embden-Meyerhof scheme as it does at a point other than the glucose phosphorylation step, would help to resolve some of these controversial issues.

EXPERIMENTAL PROCEDURES AND METHODS

Fructose [3] and glucose in amounts of 1.0 gm. per kg. were administered intravenously in a 10% solution during a period of 60 minutes to three normal adult males and five patients with diabetes mellitus. The diabetes was severe enough in all instances to require insulin. Regular insulin was given in two doses, one before breakfast and one before supper for three days, in order that, on the morning of any test, when insulin was withheld, the last dose of insulin would have been received at least 14 hours before and no significant insulin effect would be present. Fasting hyperglycemia of over 200 mg. per 100 ml. was usually present, with concomitant glycosuria. None of the subjects had any clinical signs of thiamine

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[2] This work was supported in part by a grant from the Cleveland Diabetic Fund.

[3] The fructose for intravenous use was generously supplied by Dr. Warren Cox, Jr., Medical Director of Mead Johnson & Company.

deficiency, but B complex capsules were given for three days prior to the tests so that the supplementary thiamine intake was at least 20 mg. daily. This was done to eliminate any possible effect of a subclinical thiamine deficiency on pyruvate metabolism. The previous diets were all nutritionally adequate by present-day dietary standards, and for the diabetic subjects averaged 180 gm. of carbohydrate daily. The tests were done in the post-absorptive state (overnight fast) and in the resting state (to avoid the rise in pyruvic acid after muscular exertion).

The fructose and glucose were given to the patients usually on alternate days or after an interval of one day. Since each patient received both fructose and glucose on separate occasions, the effects of fructose could be compared directly with those of glucose in the same individual. In addition, the effects in the diabetic subject could be compared with those in the non-diabetic control. Samples of peripheral venous blood were drawn before the start of the 60-minute infusion and one-half, one, one and one-half, two, and three hours after the start of the infusion. Urine samples were collected before and at intervals up to three hours during the experiment. The blood samples were analyzed for glucose, fructose, pyruvic acid, citric acid, and inorganic phosphorus; the urine samples were analyzed for glucose and fructose. The following determinations were used as indicators of intermediary metabolism and utilization of the hexose: changes in blood pyruvic acid, plasma citric acid, and serum inorganic phosphorus; the disappearance of hexose from the blood stream; and the net amount of hexose retained in the body, obtained by subtracting the number of grams excreted in the urine from the total amount given.

Blood glucose was determined by the method of Somogyi (9); blood fructose, by the method of Roe as modified by Higashi and L. Peters (10). There was no interference with the fructose determination by blood glucose levels up to 300 mg. per 100 ml. A blood glucose level of 450 mg. per 100 ml. caused an increase in the blood fructose measurement of 6 mg. per 100 ml., and a blood glucose level of 900 mg. per 100 ml. resulted in a corresponding rise of 12 mg. per 100 ml. Appropriate corrections were made on the above basis. The blood pyruvic acid was determined by the method of Friedemann and Haugen (11). Plasma citric acid was done by a combination of methods of Natelson, Pincus and Lugovoy (12) and of Taussky and Shorr (13). The Fiske and Subbarow method (14) was used for serum inorganic phosphorus.

The significance of changes in blood glucose, blood pyruvic acid, and plasma citric acid was estimated from the results of the following experiment. Blood samples from each of four normal subjects were drawn at 30 to 60 minute intervals for three and one-half hours under the same experimental conditions outlined above, except that no infusion was given. Using the initial level as a reference the mean standard deviations (σ) and their variances, in mg. per 100 ml., were as follows: for glucose, $\sigma = 3.0 \pm 1.4$; for pyruvic acid, 0.17 ± 0.037; and for citric acid, 0.15 ± 0.096.

RESULTS

A. Normal subjects. The complete data depicting the metabolic effects of intravenous glucose and fructose are shown in Tables I and II. After glucose the average rise in blood glucose above the initial level was 176 mg. per 100 ml., followed by a rapid fall to hypoglycemic levels 60 minutes after the infusion was completed, with a subsequent return to normal. After fructose, however, the average rise in blood fructose above the initial level was 81 mg. per 100 ml., which was less than one-half that obtained with glucose given in comparable amounts and at the same rate. A rise in blood glucose following fructose administration was noted in two of the three cases.

The excretion of fructose in the urine during the three hour period of the experiment was in the same range as that of glucose. On the average somewhat less than 10% of the administered fructose or glucose appeared in the urine. No threshold for fructose excretion was found, since significant fructosuria was present even at blood levels as low as 4 mg. per 100 ml.

Pyruvic acid levels rose on the average 32% or 0.31 mg. per 100 ml. above the control level after glucose;[4] but the rise after fructose was three times as great. Citric acid levels after glucose[4] fell slightly in the two cases studied; but after fructose citric acid rose in all three cases.

Serum inorganic phosphorus fell invariably after fructose administration in normal subjects. Serum phosphorus was not measured after glucose, since the drop in inorganic phosphorus has been so well established by others.[5]

The administration of fructose to a fourth nondiabetic subject produced similar changes: rises in pyruvic acid (2.09 mg. per 100 ml.), citric

[4] In this laboratory a larger series of normal subjects comprising 10 individuals given 1 gm. of glucose per kg. intravenously in a period of 60 minutes showed an average maximal increase in pyruvic acid of 35.7% or 0.36 mg. per 100 ml. and an average fall of 20.5% or 0.43 mg. per 100 ml. in citric acid.

[5] Forsham and Thorn (16) found an average fall of 25% in the serum phosphorus of normal subjects after intravenous administration of 0.5 gm. of glucose per kg. in 30 minutes. In one of our normal subjects (J. C.) the serum phosphorus fell 27% after 1 gm. of glucose per kg. in 60 minutes given at a date subsequent to the experiment listed in Table I.

TABLE I

Comparison of effects of intravenous glucose and fructose in normal subjects
(1 gm. per kg. as 10% solution in 60 minutes)

Subject	Glucose					Fructose					
	Date	Time	Blood glucose	Blood pyruvic acid	Plasma citric acid	Date	Blood fructose	Blood pyruvic acid	Plasma citric acid	Serum inorg. P	Blood glucose
		min.	*mg. %*	*mg. %*	*mg. %*		*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
J. O.	8/17/50	0	73	1.20		5/3/51	2	1.31	1.73	3.28	85
Age		30	234	1.15			58	2.81	2.12	2.50	88
30		60	263	1.36			68	2.78	2.12	2.48	110
Wt.		90	79	1.28			26	1.71	2.24	2.99	84
61 kg.		120	42	1.15			10	1.04	2.04	2.96	74
		150	50	1.50*							
		180					5	1.07	1.71	2.93	76
J. C.	9/5/50	0	74	0.80	2.08	5/7/51	1	1.15	2.16	2.87	78
Age		30	213	1.12	1.89		90	1.95	2.12	1.77	90
30		60	202	1.23	1.99		97	2.19	2.24	1.77	94
Wt.		90	54	0.96	2.18		27	1.68	2.48	2.82	61
67 kg.		120	39	0.88	2.08		12	1.28	2.10	2.92	71
		180	68	1.47*	2.05		5	0.88	1.86	3.20	81
W. D.	9/28/50	0	79	1.20	2.86	3/15/51	1	0.91	2.18	3.15	89
Age		30									
28		60	283	1.55	2.80		144†	1.76	2.30	1.80	85
Wt.		90	98	1.31	2.68		34	1.63	2.53	3.26	85
80 kg.		120	42	1.28	2.38		14	1.23	2.47	3.04	61
		180	71	1.28	2.58		4	0.96	1.71	2.99	71
		240	88	0.75	2.42		1	1.10	1.38	2.99	76

* A second rise in pyruvic acid accompanying the hypoglycemia which follows intravenous glucose administration occurs in the majority of cases (15).

† Infusion stopped at 30 minutes; restarted and speeded up at end; hence very high value at 60 minutes. Value omitted from average.

acid (0.57 mg. per 100 ml.), and glucose (9 mg. per 100 ml.); and a fall in serum inorganic phosphorus (0.37 mg. per 100 ml.). Two gm. of fructose were excreted in the three hour period. A glucose experiment was not performed in this subject, eliminating the opportunity for a direct comparison of the effects.

Figure 1 compares graphically the changes after glucose and fructose in a typical experiment (Subject J. C.).

B. Diabetes mellitus. The data obtained in five cases of diabetes mellitus have been listed in Tables III and IV. The changes in blood glucose following intravenous glucose were characteristic of diabetes mellitus, with marked hyperglycemia and a delayed fall. In direct contrast, the blood disappearance curve for fructose was, in every instance, essentially identical with that found in the normal group. Corresponding to these similar blood levels, the excretion of fruc-

TABLE II

Three hour excretion of hexose in urine after intravenous glucose and fructose in normals

Subject	Amount of hexose injected	After glucose			After fructose				
		Date	Volume of urine	Glucose	Date	Volume of urine	Total hexose	Glucose	Fructose
	gm.		*ml.*	*gm.*		*ml.*	*gm.*	*gm.*	*gm.*
J. O.	61				5/3/51	640	3.1	0.2	2.9
J. C.	67	9/5/50		5.5	5/7/51	740	4.3	0	4.3
		5/18/51	1,250	3.4					
W. D.	80	9/28/50	1,010	9.3	3/15/51	946	5.9	0.1	5.8

tose in the urine of the diabetic patient was in the same range (2.0 to 5.7 gm.) as in the normal. The diabetic subject differed from the normal in that, in all but one instance, there was a more marked rise in blood glucose following fructose. This rise was only roughly correlated with the severity of the diabetes as measured by the height of the fasting blood sugar and the delay in the return of the blood sugar to the fasting level after glucose. This blood glucose elevation caused an increased excretion of glucose, but in the three cases where excretion could properly be compared because of the approximate equality of the initial blood sugars (M. G., A. B., and

TABLE III

Comparison of effects of intravenous glucose and fructose in diabetes mellitus
(1 gm. per kg. as 10% solution in 60 minutes)

Subject	Glucose						Fructose					
	Date	Time	Blood glucose	Blood pyruvic acid	Plasma citric acid	Serum inorg. P	Date	Blood fructose	Blood pyruvic acid	Plasma citric acid	Serum inorg. P	Blood glucose
		min.	*mg. %*	*mg. %*	*mg. %*	*mg. %*		*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
M. G.	4/1/51	0	230	1.57	2.77	3.78	3/30/51	2	1.63	2.64	3.98	220
Age		30		1.20	2.97	3.65					3.34	
45		60	533	1.07	2.94	3.87		89	4.17	3.66	3.29	259
Wt.		90	470	1.44	3.01	3.56		35	4.01	3.57	3.92	253
86 kg.		120	428	1.74	3.31	3.78		19	3.53	3.66	3.63	254
		180	344	1.84	2.84	3.51		8	2.14	3.51	3.42	236
		0					4/3/51	4	1.66	2.65	4.00	177
		30									3.31	
		60						78	3.80	3.10	3.39	265
		90						34	3.56	3.30	3.97	258
		120						16	2.54	3.18	3.48	244
		180						6	2.06	3.03	3.45	228
A. B.	4/14/51	0	236	1.12	2.38	2.18	4/12/51	4	1.47	2.68	2.21	222
Age		30				2.10					1.41	
53		60	568	1.23	2.24	2.01		82	4.12	2.88	1.43	243
Wt.		90	443	1.42	2.36	2.12		30	3.00	2.76	1.78	244
55 kg.		120	389	1.55	2.28	1.95		20	2.49	2.59	1.75	228
		180	297	1.28	1.90	1.95		8	1.42	2.00	1.86	224
M. H.	4/23/51	0	368	0.80		3.71	4/19/51	1	1.34	2.02	4.46	306
Age		30	572	0.94		3.74		57	1.66	1.99	3.26	408
26		60	640	1.02		3.48		64	1.95	2.02	3.42	438
Wt.		90	588	1.10		3.43		22	1.74	2.41	4.18	508
55 kg.		120	586	1.10		3.60		18	1.68	1.93	4.18	528
		180	540	1.04		3.40		5	1.44	1.90	4.28	525
		0					4/24/51	2	1.36	2.01	3.82	400
		30						61	2.40	2.01	3.20	484
		60						61	2.24	2.22	3.14	531
		90						22	2.00	2.04	3.68	573
		120						11	1.74	2.18	3.43	570
		180						6	1.34	1.98	3.63	558
J. R.	4/25/51	0	226	0.83	2.46	3.68	4/26/51	4	1.31	2.38	2.78	224
Age		30	409	1.26	2.51	2.92		71	1.98	3.35	2.34	245
60		60	462	1.26	2.51	2.89		70	1.90	2.76	2.29	293
Wt.		90	382	1.23	2.30	3.20		28	1.58	2.80	2.45	304
53 kg.		120	346	1.23	2.51	2.92		19	1.60	2.76	2.55	279
		180	282		2.49	2.86		10	1.31	2.91	2.63	264
	4/27/51	0	226	1.20								
		90	374	1.20								
		180	278	1.42								
E. R.	6/14/51	0	162	1.02	2.58	3.60	6/12/51	3	1.02	2.82	3.46	126
Age		60	512	1.23	2.47	3.24		90	2.78	3.26	3.02	185
52		90	415	1.58	2.72	3.27		35	2.36	2.94	3.34	155
Wt.		120	358	1.39	2.38	2.88		16	1.52	2.30	3.28	131
68 kg.		180	256	1.02	2.08	2.79		7	1.36	2.26	3.14	133

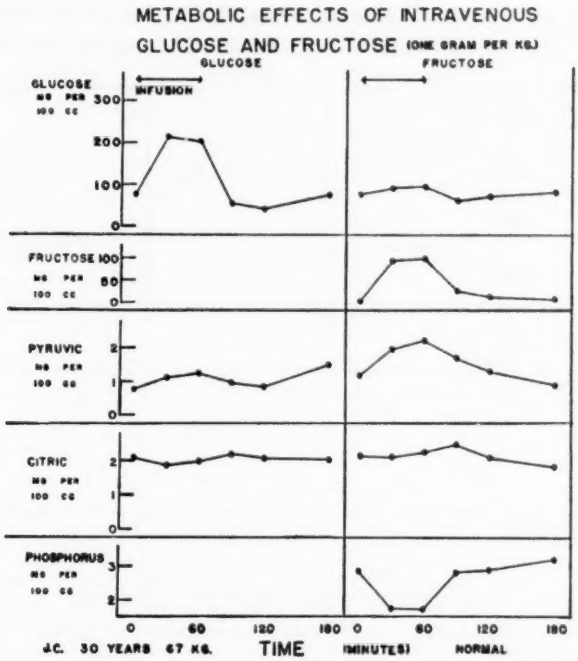

FIG. 1. COMPARISON OF THE METABOLIC EFFECTS OF INTRAVENOUS GLUCOSE AND FRUCTOSE IN A *NORMAL* SUBJECT (J. C.)

TABLE IV

Three hour excretion of hexose in urine after intravenous glucose and fructose in diabetes mellitus

Subject	Amount of hexose injected	After glucose				After fructose					
		Date	FBS*	Volume of urine	Glucose	Date	FBS*	Volume of urine	Total hexose	Glucose	Fructose
	gm.		*mg. %*	*ml.*	*gm.*		*mg. %*	*ml.*	*gm.*	*gm.*	*gm.*
M. G.	80	4/1/51	230	870	26.1	3/30/51	220	1,095	12.2	6.5	5.7
						4/3/51	177	767	9.8	4.4	5.4
A. B.	55	4/14/51	236	599	23.7	4/12/51	222	787	9.7	5.9	3.8
M. H.	55	4/23/51	368	864	38.5	4/19/51	306	830	23.7	21.7	2.0
						4/24/51	400	945	37.3	35.1	2.2
J. R.	53	4/25/51	226	596	29.7	4/26/51	224	570	19.7	16.5	3.2
		4/27/51	226	606	27.4						
E. R.	68	6/14/51	162	850	15.9	6/12/51	126	868	3.8	0.1	3.7

* Fasting blood sugar.

J. R.) 25%, 25%, and 58% as much glucose was excreted after fructose as after glucose. When the urinary loss of fructose was added to that of glucose in the fructose experiments, the advantage was still apparent, with a saving of one-third to one-half of the total hexose. In the most severe diabetic subject (M. H.) a direct quantitative comparison could not be made because the fasting blood sugars were not in the same range. However, less glucose (35.1 gm. as com-

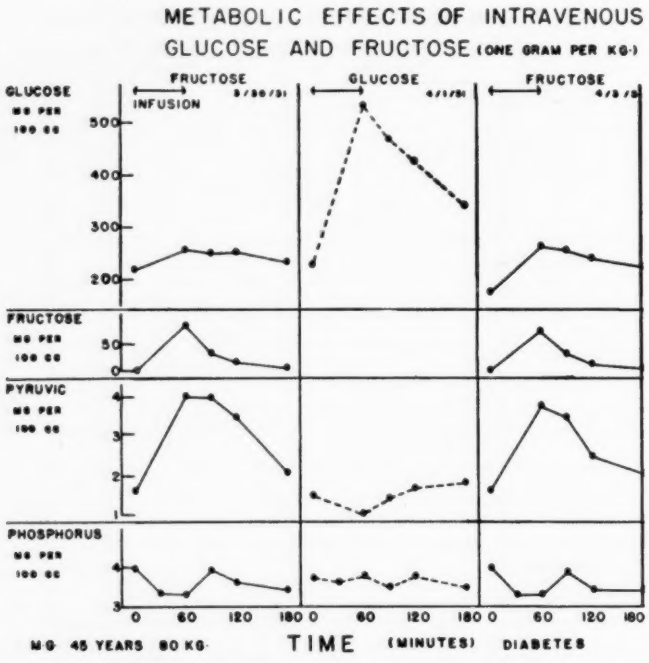

FIG. 2. COMPARISON OF THE METABOLIC EFFECTS OF INTRAVENOUS GLUCOSE AND FRUCTOSE IN A *DIABETIC* SUBJECT (M. G.), WHOSE DIABETES WAS OF RECENT ONSET AND STABLE IN TYPE

pared with 38.5 gm.) was lost after the fructose infusion when the initial blood sugar level was 400 mg. per 100 ml. than after the glucose injection when the initial blood sugar level was 32 mg. per 100 ml. less.

One of the striking differences in the metabolism of fructose and glucose in the diabetic subject was shown by the changes in blood pyruvic acid. After glucose, the rise in pyruvic acid was similar in magnitude to that observed in normal individuals; but it was delayed, and usually occurred during the period when the blood sugar was falling; in two instances a significant rise did not take place.[6] After fructose, however, the rise occurred just as promptly as in the normal subject. In addition, the magnitude of the pyruvic acid rise in the diabetic patient after fructose was as great as, or even greater than, the rise found in the normal subject. In four of the

[6] The changes in blood pyruvic acid in diabetic patients following the *oral* administration of glucose have been studied by several investigators. Bueding, Wortis and Fein (7) found either no increase in pyruvic acid or a rise (in four of 10 cases) which was smaller and had a tendency to occur later than in normal subjects. Klein (17) observed a small and delayed rise. Horwitt, Hills and Kreisler (18) reported a rise which was of normal magnitude but appeared later. Root, Stotz and Carpenter (3) found that two of four clinically mild diabetic patients showed a pyruvic acid rise after glucose ingestion while only two of 20 cases with moderate or severe diabetes mellitus showed a significant rise which came only after one and one-half hours. Interpretation of the results of the last three studies is difficult since at least some of the subjects had received protamine zinc insulin within 14, 20 or 12 hours, respectively, of the tests. Miller and his associates (19) administered 1 gm. of glucose per kg. intravenously to three human subjects with diabetes and observed no significant rise in blood pyruvic acid. Bueding, Fazekas, Herrlich and Himwich (20) reported no increase in pyruvic acid in depancreatized dogs after the intravenous administration of 2.0 gm. of glucose per kg. These results suggest that in the presence of a diminished insulin supply (as in some human diabetic patients) either a smaller than normal or a delayed rise in pyruvic acid may occur or both. In the complete absence of insulin

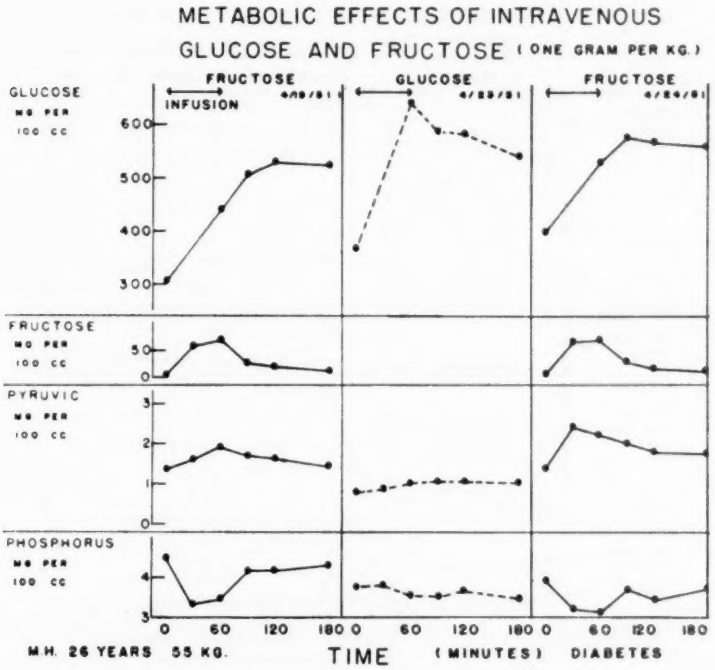

FIG. 3. COMPARISON OF THE METABOLIC EFFECTS OF INTRAVENOUS GLUCOSE AND FRUCTOSE IN A *DIABETIC* SUBJECT (M. H.), WHOSE DIABETES WAS OF LONG DURATION AND WAS LABILE IN TYPE

cases (M. G., A. B., M. H., and E. R.) there was an inverse relationship between the rise in pyruvic acid and the rise in blood glucose after fructose administration. For example, in case A. B. a rise of 2.65 mg. per 100 ml. was associated with a rise of 22 mg. per 100 ml. in the blood glucose; whereas in case M. H. a rise of only 0.61 mg. per 100 ml. occurred when the blood glucose rose 222 mg. per 100 ml.

(as in the depancreatized dog), on the other hand, no pyruvic rise at all was found after glucose administration. When, however, a large enough dose of glucose was given to the depancreatized dog to raise the blood level to between 700 and 1,000 mg. per 100 ml. a delayed rise in pyruvic acid occurred (20), indicating that the total dose and the rate of administration of glucose may also influence the entrance of glucose into the reactions of the Embden-Meyerhof scheme. Soskin and Levine (21) also found that the rate of utilization of glucose in the depancreatized, eviscerated dog was dependent upon the level of blood glucose.

Plasma citric acid levels were determined in four diabetic subjects after glucose administration. The changes in citric acid were variable—in two cases there was a fall; in one, a rise; and in the fourth case there was no significant change. These results were similar to those reported by Pincus, Natelson, and Lugovoy (22). Following fructose administration, there was a significant rise in citric acid in five out of seven tests; these results approached those obtained after fructose in non-diabetic subjects.

It has long been established that in diabetes the decrease in serum inorganic phosphorus after the administration of glucose is diminished or abolished (16, 23). Insignificant falls of 0.27, 0.23, and 0.31 mg. per ml. were found in the first three cases, and again these occurred late; whereas after fructose in the same three cases the falls in phosphorus were 0.69 and 0.69 (M. G.), 0.80 (A. B.), and 1.20 and 0.68 mg. per 100 ml.

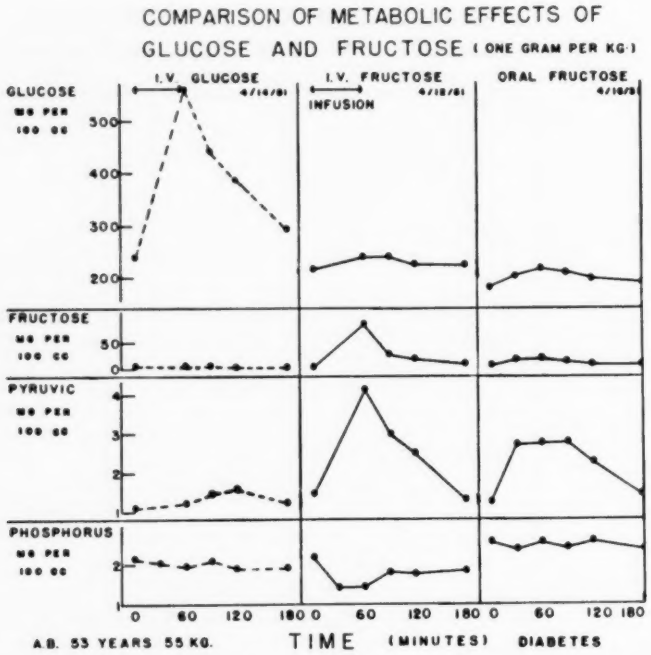

FIG. 4. COMPARISON OF THE METABOLIC EFFECTS OF INTRAVENOUS GLUCOSE AND FRUCTOSE IN A *DIABETIC* SUBJECT (A. B.)

The third column contrasts the effects of orally administered fructose.

(M. H.). In the fourth and fifth cases phosphorus fell after both glucose and fructose.

Figures 2 and 3 compare graphically the changes in blood glucose, pyruvic acid, and serum inorganic phosphorus in two different types of diabetes. The diabetes was of recent onset in case M. G. (Figure 2) who was of the older, obese type; while the diabetes had been present 12 years in case M. H. (Figure 3), representing the younger, more severe and labile group.

C. Oral fructose versus intravenous fructose in the diabetic subject. In one case (A. B.) an opportunity was available to compare the effects of fructose given by mouth with the same amount given intravenously (1 gm. per kg.). The results are shown graphically in Figure 4. After oral administration, the highest blood fructose level was only 17 mg. per 100 ml., contrasted with a peak of 82 mg. per 100 ml. after the intravenous route was used, and even at 120 minutes the two blood levels were 8 and 20 mg. per 100 ml., respectively. Associated with the much lower blood level of fructose, the elevation of blood glucose was more than twice as great (51 mg. per 100 ml. as compared with 22) and remained higher for a longer period of time. Part of the fructose, therefore, must have been converted to glucose in its passage through the bowel wall and the liver. Despite a lower initial blood sugar in the oral experiment, there was an augmented excretion of glucose in the urine (13.6 gm. as compared with 5.9 gm.) but only a minute amount of fructose. The rise in pyruvic acid after oral fructose was only three-fifths that found after the intravenous route was used, and no significant fall in inorganic phosphorus was found. It is evident from these findings that in diabetes fructose is metabolized in much greater quantities when given directly into the blood stream, because less of it is converted to glucose.

DISCUSSION

The results of our studies indicate that *1*) the metabolism of fructose differs from that of glucose in both the normal and diabetic subject, and *2*) the metabolism of fructose in the person with diabetes is similar to that in the normal person, even in the absence of insulin.

The observation that, in normal individuals, the rise in the level of blood fructose following fructose administration is less than the rise in blood glucose after the injection of the same amount of glucose proves that fructose is removed from the blood more rapidly than glucose during the period of infusion. The rise in blood glucose following fructose administration indicates that part of the fructose is converted to glucose. Another fraction of the fructose is metabolized by way of the Embden-Meyerhof series of reactions and the tricarboxylic acid cycle. This is indicated by the rises in pyruvic and citric acid. Glycogen formation undoubtedly accounts for another part of the fructose removed from the blood (*24*). The fall in phosphorus after fructose reflects the process of phosphorylation which is involved in the metabolism of both hexoses studied.

Fructose produces a much greater rise in pyruvic acid than does glucose. Two possible explanations can be offered: *1*) fructose enters the metabolic scheme closer to pyruvic acid, at a different point from glucose, *2*) fructose enters at the same point as glucose (*i.e.*, at glucose 6-PO_4 by way of fructose 1-PO_4 [25]) at a more rapid rate, or is metabolized more quickly. The data obtained in these experiments do not permit us to choose between the two possibilities. That the elevation of pyruvic acid in diabetic subjects after fructose is not due to a decreased rate of removal, is indicated by the studies of Bueding and associates (*20*), which showed that there was no difference in the rate of disappearance of injected pyruvate from the blood in normal and depancreatized dogs. Oxidative metabolism of fructose seems to be unimpaired, according to the recent report of Chernick and Chaikoff (*26*). These workers found that the oxidation of C^{14}-fructose to CO_2 proceeded at normal rates in liver slices from alloxan diabetic rats. The greater rise in citric acid after fructose administration is still another indication of the difference between the metabolism of fructose and glucose.

Although the blood levels of fructose were lower than those of glucose, similar quantities of the two substances were excreted in the urines of the normal subjects. This observation can be explained by the fact that fructose appears in the urine at low blood levels, while glycosuria occurs only at higher levels of blood glucose.

The individual with diabetes mellitus handles fructose like a normal subject, as shown by the similar elevations of fructose, pyruvic acid, and (in several cases) citric acid; the falls in phosphorus and the total amount of fructose excreted in the urine are also comparable. The greater rise in blood glucose following fructose administration to the diabetic patient, as compared with the normal individual, probably reflects the diminished ability to utilize glucose, and possibly a greater conversion of fructose to glucose.

Fructose is better utilized by the diabetic when administered intravenously than when given orally, presumably because of its greater conversion to glucose in the intestine and liver when given by the latter route.

The difference in the metabolism of fructose and glucose in subjects with diabetes mellitus is much more marked than the difference in normal subjects. The diabetic individual appears to metabolize fructose at a normal rate, but glucose utilization is impaired. In these studies diminished glucose utilization is shown by the greater rise in glucose, delayed rise in pyruvic acid, diminished fall in phosphorus, and the increased excretion of glucose as compared to the changes found in the normal subjects.

That mechanisms exist to explain this difference in the metabolism of fructose and glucose is shown by the findings of Cori and Cori (*27*). In eviscerated rats insulin accelerated the disappearance of injected glucose, but not of injected fructose (*27*). This is now explained by the existence of separate "kinases" in liver and muscle for the disposal of fructose and glucose, respectively; apparently insulin acts only on the glucokinase of muscle (*25, 28, 29*). A similar conclusion with respect to liver arises from the recent experiments of Chernick and Chaikoff (*26*).

The present observations on the utilization of

glucose and fructose by normal and diabetic subjects are consistent with the theory that the phosphorylation of glucose to glucose-6-phosphate is impaired in diabetes, while the phosphorylation of fructose, which is under the influence of a separate hexokinase, at least in liver and muscle, and is uninfluenced by insulin, proceeds at a normal rate.

SUMMARY AND CONCLUSIONS

The demonstration by Cori that insulin influences the phosphorylation of glucose and that separate hexokinases for glucose and fructose exist in liver and muscle, raised the possibility that fructose might be better utilized than glucose in diabetic subjects. Accordingly, similar amounts of fructose and glucose were administered intravenously to five diabetic subjects. Three normal subjects were used for comparison.

In the ***normal*** subjects fructose produced a much greater rise in blood pyruvic acid, and a rise rather than a fall in plasma citric acid, as compared with glucose. The fructose disappeared more rapidly from the blood stream during the period of the infusion, since the blood level of fructose increased only half as much as the level of glucose. Similar amounts (less than 10%) of fructose and glucose appeared in the urine.

In the *diabetic* subjects, in the absence of insulin, fructose produced the same rise in pyruvic acid as was found in the normal subjects, while glucose produced either a slight or delayed rise. Increases in plasma citric acid after fructose administration were similar to those in the normal individuals. The blood disappearance curve of fructose and the amount of fructose excreted in the urine in patients with diabetes were the same as in normal subjects. Fructose administration invariably produced a fall in serum inorganic phosphorus, while a fall did not usually occur after glucose. The increase in blood glucose after fructose injection was higher in diabetic subjects than in non-diabetic individuals. In one patient with diabetes oral administration of fructose, as compared with intravenous administration, produced a much lower level of blood fructose, a greater rise in blood glucose, a smaller rise in blood pyruvic acid, and a greater excretion of glucose.

These studies indicate that *1*) the metabolism of fructose differs from that of glucose in both the normal and diabetic subject, and *2*) the metabolism of fructose in the person with diabetes is similar to that in the normal person, even in the absence of insulin. The observations are consistent with the theory that the phosphorylation of glucose to glucose-6-phosphate is impaired in diabetes, while the phosphorylation of fructose, which is uninfluenced by insulin, proceeds at a normal rate.

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REFERENCES

1. von Noorden, C., Die Zuckerkrankheit und ihre Behandlung. A. Hirschwald, Berlin, 1910, 5th edition.
2. Joslin, E. P., Root, H. F., White, P., and Marble, A., The Treatment of Diabetes Mellitus. Lea and Febiger, Philadelphia, 1946, 8th edition, p. 356.
3. Root, H. F., Stotz, E., and Carpenter, T. M., The respiratory quotient and blood pyruvate and lactate responses after oral ingestion of glucose and fructose in diabetes mellitus with and without insulin. Am. J. M. Sc., 1946, **211**, 189.
4. Stewart, C. P., and Thompson, J. C., The conversion of laevulose and fatty acid into glucose. Biochem. J., 1941, **35**, 245.
5. Bollman, J. L., and Mann, F. C., The physiology of the liver. XIX. The utilization of fructose following complete removal of the liver. Am. J. Physiol., 1931, **96**, 683.
6. Weichselbaum, T. E., Elman, R., and Lund, R. H., Comparative utilization of fructose and glucose given intravenously. Proc. Soc. Exper. Biol. & Med., 1950, **75**, 816.
7. Bueding, E., Wortis, H., and Fein, H. D., Pyruvic acid metabolism in diabetes mellitus. Am. J. M. Sc., 1942, **204**, 838.
8. Price, W. H., Cori, C. F., and Colowick, S. P., The effect of anterior pituitary extract and of insulin on the hexokinase reaction. J. Biol. Chem., 1945, **160**, 633.
9. Somogyi, M., Determination of blood sugar. J. Biol. Chem., 1945, **160**, 69.
10. Higashi, A., and Peters, L., A rapid colorimetric method for the determination of inulin in plasma and urine. J. Lab. & Clin. Med., 1950, **35**, 475.
11. Friedemann, T. E., and Haugen, G. E., Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem., 1943, **147**, 415.
12. Natelson, S., Pincus, J. B., and Lugovoy, J. K., Microestimation of citric acid; a new colorimetric reaction for pentabromoacetone. J. Biol. Chem., 1948, **175**, 745.

13. Taussky, H. H., and Shorr, E., A microcolorimetric method for the determination of citric acid. J. Biol. Chem., 1947, **169**, 103.
14. Fiske, C. H., and Subbarow, Y., The colorimetric determination of phosphorus. J. Biol. Chem., 1925, **66**, 375.
15. Taft, H. P., Miller, M., Drucker, W. R., and Craig, J. W., Metabolic and endocrine changes with hypoglycemia. J. Clin. Endocrinol., 1951, **11**, 752.
16. Forsham, P. H., and Thorn, G. W., Changes in inorganic serum phosphorus during the intravenous glucose tolerance test as an adjunct to the diagnosis of early diabetes mellitus. Proc. Am. Diabetes Assoc., 1949, **9**, 99.
17. Klein, D., The effects of administration of glucose and insulin on blood pyruvate and lactate in diabetes mellitus. J. Biol. Chem., 1942, **145**, 35.
18. Horwitt, M. K., Hills, O. W., and Kreisler, O., Lactic and pyruvic acids in the blood after glucose and exercise in diabetes mellitus. Am. J. Physiol., 1949, 156, 92.
19. Miller, M., Bueding, E., Strauch, R. O., Owens, J., and Woodward, H., Studies on pyruvate and citrate metabolism in man and animal. Proc. Am. Diabetes Assoc., 1949, **9**, 83.
20. Bueding, E., Fazekas, J. F., Herrlich, H., and Himwich, H. E., Effect of insulin on pyruvic acid formation in depancreatized dogs. J. Biol. Chem., 1943, **148**, 97.
21. Soskin, S., and Levine, R., A relationship between the blood sugar level and the rate of sugar utilization, affecting the theories of diabetes. Am. J. Physiol., 1937, **120**, 761.
22. Pincus, J. B., Natelson, S., and Lugovoy, J. K., Response of citric acid levels to oral administration of glucose. II. Abnormalities observed in the diabetic and convulsive state. J. Clin. Invest., 1948, **27**, 450.
23. Bolliger, A., and Hartman, F. W., Observations on blood phosphates as related to carbohydrate metabolism. J. Biol. Chem., 1925, **64**, 91.
24. Cori, C. F., The fate of sugar in the animal body. III. The rate of glycogen formation in the liver of normal and insulinized rats during the absorption of glucose, fructose and galactose. J. Biol. Chem., 1926, **70**, 577.
25. Cori, G. T., Ochoa, S., Slein, M. W., and Cori, C. F., The metabolism of fructose in liver. Isolation of fructose-1-phosphate and inorganic pyrophosphate. Biochim. & Biophys. Acta, 1951, 7, 304.
26. Chernick, S. S., and Chaikoff, I. L., Two blocks in carbohydrate utilization in the liver of the diabetic rat. J. Biol. Chem., 1951, **188**, 389.
27. Cori, C. F., and Cori, G. T., Fate of glucose and other sugars in the eviscerated animal. Proc. Soc. Exper. Biol. & Med., 1928–29, **26**, 432.
28. Slein, M. W., Cori, G. T., and Cori, C. F., A comparative study of hexokinase from yeast and animal tissues. J. Biol. Chem., 1950, **186**, 763.
29. Cori, C. F., Personal communication.

APPENDIX I

TABLE V

Clinical data on diabetic subjects

Subject	Hospital no.	Age	Sex	Duration of diabetes	Insulin dose	Other data
					(units per day)	
M. G.	607–250	45	F	5 wks.	40	Obesity, hypertension
A. B.	608–037	53	F	1 yr.	65	None
M. H.	201–785	26	M	12 yrs.	50	Intermittent hypertension, neuropathy
J. R.	102–976	59	M	7 yrs.	30	Diabetes developed after partial pancreatectomy for islet cell adenoma, 1944
E. R.	619–071	53	F	2 yrs.	25	Mild hypertension, retinal exudates

APPENDIX II

After the completion of this manuscript the paper of A. Pletscher, H. Fahrländer, and H. Staub, Helvet. Physiol. et Pharmacol. Acta, 1951, **9**, 46, came to our attention. Fructose and glucose (0.5 gm. per kg. per 20 min.) were administered intravenously to normal and diabetic subjects. Their findings with regard to the similarity of the blood fructose disappearance curve and the rise in blood pyruvic acid after fructose injection were similar to those reported here. Plasma citric acid and hexose excretion were not determined.

TOTAL OXYGEN CONSUMPTION AND METABOLIC RATE OF PATIENTS IN DIABETIC ACIDOSIS[1]

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There is little information on the metabolic rate of patients in severe acidosis resulting from uncontrolled diabetes mellitus, and among the few studies which have been reported, there is no general uniformity of results. It has usually been concluded that there is no striking change in metabolic rate of patients in diabetic acidosis. Recent studies in this laboratory do not confirm these reports, and the changes found have been consistent and definite.

Many of the early studies included patients who were in mild or questionable acidosis, and comparison of their metabolic rates to normal were made on a simple height : weight relationship rather than on the more valid DuBois standards (1) which comprehend body surface area. Allen and DuBois (2) in 1916, Wilder and associates (3) in 1922, and Holton (4) in 1924, all presented complete reviews, including the studies of Hédon (5), Joslin and Bennedict (cited in [2]) and Bernstein and Falta (6). These reviewers agreed that the previous investigators obtained variable results and that the most reasonable conclusion was that there is no significant or striking change of the metabolic rate of patients in diabetic acidosis as compared to normal. Richardson and Mason (7) studied a case of diabetic coma in a "respiration calorimeter" and found an increase in the basal metabolic rate of 11 per cent. Duncan (8) as late as 1947 listed diabetic acidosis under the category of miscellaneous disorders which caused clinically unimportant changes in the basal metabolic rate. Joslin and associates (9) agreed with all previous reviewers and quoted the statement of DuBois (10) : "The role of acidosis in the metabolism of a diabetic is extremely complicated, and I doubt if it will ever be definitely settled. At the present time it seems as if a severe acidosis might cause a moderate increase in metabolism in some cases of diabetes."

METHOD

Each patient was studied immediately upon admission to the ward and before any treatment was given. A general statement regarding the procedure was given to the patients so as to prevent undue anxiety associated with holding a mask over the face. The method of indirect calorimetry with an open circuit was used. By means of a well fitting face mask, the patient breathed room air. His total expired air was collected in a calibrated Tissot spirometer. All patients were hyperventilating so that it required a collection of but two to four minutes to fill the spirometer completely. A preliminary collection was first made to wash out the spirometer and to accustom the patient to the procedure. The total gas expired per unit of time was calculated. Corrections were made for barometric and vapor pressure and temperature to reduce all figures to standard conditions. Samples of the gas in the spirometer were transferred immediately to Bailey bottles and analysed in a Haldane apparatus for oxygen and carbon dioxide content. Oxygen consumption per minute and basal metabolic rate were calculated from these data by standard formulae (11).

RESULTS

Table I summarizes the results obtained on patients in diabetic acidosis, two of whom were also studied after therapy when they were no longer in acidosis. The data presented include all patients upon whom respiratory studies were performed. Every patient proved to have a significant hyperglycemia and hyperketonemia diagnostic of uncontrolled diabetes mellitus. It can be seen that in every case but one, the basal metabolic rate was significantly greater than normal, the highest being + 77 and the average being + 41.4. Patient F. P., having a basal metabolic rate of + 19 before therapy, returned to + 7 when her diabetes was

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controlled and patient J. M. decreased from + 25 to + 3.

Seven non-diabetic patients studied in the same way were used as controls to rule out any systematic errors in the methods employed or in the effect of the procedure itself on the metabolic rate. The results vary greatly since these patients were not true normals, all being hospitalized for various disorders; they were selected on the basis of near normality, being afebrile and without known acute disease and were ready for discharge from the hospital for their particular illnesses. They were studied under standard basal conditions. Four patients had positive values and three negative, the average being − 2.7. No systematic deviation was found in the control group, and there was a highly significant difference in the basal metabolic rate between the control group and the patients in diabetic acidosis ($P < 0.01$).

Since all of the patients in acidosis were hyperventilating, a control experiment was performed to determine the effect of moderate hyperventilation on oxygen consumption and metabolic rate. Subjects were healthy young physicians or medical students, not necessarily fasting. Subjects rested from 30 to 45 minutes prior to collection of expired gas. A resting volume was then collected for periods of two to four minutes. Oxygen concentration in the expired air was calculated from the oxygen tension measured by a Pauling oxygen meter which had been previously calibrated against gas mixtures of known concentration—these gas mixtures having been analyzed by a Scholander micro-gas analysis apparatus. Oxygen consump-

TABLE I

Basal metabolic rate in diabetic acidosis compared to non-diabetic controls

Patient	Age (yrs.)	Sex	Height (cm.)	Weight (kg.)	Arterial blood CO_2 content (vol. %)	Arterial blood pH	Mental state*	Respiratory volume (liters/min.)	R.Q.	Oxygen consumption (cc./min.)	B.M.R.
Patients in diabetic acidosis—before therapy											
F. P.	60	F	173	110	25.0	7.39	1	10.1	0.63	313	+19
C. C.	50	M	185	96	8.3	6.94	2	22.6	0.56	386	+31
J. S.	36	M	170	73	20.4	7.27	1	15.1	0.62	434	+62
N. D.	53	F	155	61	14.2†	7.28†	2	13.4	0.56	280	+57
J. M.	18	M	170	50	10.4	7.12	2	15.3	0.63	289	+25
S. B.	49	M	168	52	7.8	7.14	1	20.8	0.72	283	+35
G. F.	19	M	173	47	14.6	7.18	1	13.9	0.76	303	+27
N. C.	43	M	170	50	29.6	7.21	1	8.9	0.63	225	+ 3
A. O.	38	F	165	67	8.6	7.19	2	21.1	0.61	351	+54
H. J.	50	M	163	56	13.0†	7.13†	1	19.2	0.64	352	+65
H. R.	35	M	180	60	11.1	7.14	2	18.1	0.49	484	+77
Mean	44		170	66	14.8	7.18		16.2	0.62	336	+41.4 ± s.e. 6.9
Patients in diabetic acidosis—after therapy											
F. P.	60	F	173	110	66.2	7.43	1	7.7	0.80	270	+ 7
J. M.	18	M	170	50	47.5	7.41	1	7.6	0.81	228	+ 3
Hospital patients without diabetes											
R. S.	19	M	168	77	53.2	7.49	1	5.3	0.65	308	+12
L. S.	31	M	180	67	50.6	7.47	1	5.3	0.72	153	−41
T. C.	34	M	170	61	48.6	7.22	1	9.2	0.88	272	+11
M. J.	38	F	155	43	49.8	7.46	1	8.1	1.08	149	−12
B. F.	58	M	170	117	52.0	7.42	1	9.6	0.76	410	+37
E. L.	47	F	157	73	52.3	7.39	1	4.0	0.73	148	−36
A. D.	38	M	168	50	53.8	7.49	1	5.9	0.68	250	+10
Mean	38		167	70	51.5	7.42	1	6.8	0.79	241	−2.7 ± s.e. 10.7

* Mental state is described as: grade 1, normal; grade 2, lethargic, confused.
† Venous blood.

TABLE II

The effect of moderate hyperventilation on oxygen consumption and metabolic rate

Subject	Age	Sex	Height	Weight	Respiratory volume	Oxygen consumption		B.M.R.
	yrs.		*cm.*	*kg.*	*liters/min.*	*cc./min.*	*per cent change*	
(L. S.) C.	29	M	171	77	5.8	230		−11
H.					14.3	204	−11	−22
(J. K.) C.	28	M	177	73	5.0	191		−26
H.					7.3	225	+18	−14
(R. W.) C.	29	M	176	70	5.2	243		− 4
H.					14.6	262	+ 8	+ 3
(A. V.) C.	28	M	189	73	6.7	256		− 5
H.					14.2	233	− 9	−14
(B. K.) C.	26	M	181	70	5.8	272		+ 4
H.					11.9	267	− 2	+ 2
Means C.	28	M	179	73	5.7	238.4		− 8.4
H.					12.5	238.2		− 9.0

Note: B.M.R. calculated on basis of 4.825 calories per liter of oxygen.
C. = Control.
H. = Hyperventilation.

tion was calculated from the difference between (a) the product of the expired gas volume and 20.93 per cent (this latter figure taken as the oxygen concentration in room air) and (b) the product of expired gas volume and the oxygen concentration of expired air as measured on the Pauling meter. No attempt was made to correct expired gas volume to inspired volume since in a series of preliminary experiments with expired gas volume analyzed by a Scholander gas analyzer, the correction amounted to only + 1 per cent of expired volume. The maximal error in the estimation of oxygen consumption is ± 10 per cent, and the average error is ± 5 per cent.

Hyperventilation was entirely voluntary, and subjects attempted to regulate their respiratory rate in such a manner as to keep it 1.5 to 3 times the resting rate. Two subjects hyperventilated just to the point of vertigo. Hyperventilation lasted from six to 10 minutes.

The results as shown in Table II indicate that the *mean* respiratory volume was increased 2.2 times in hyperventilation over the *mean* resting volume; however, the *mean* oxygen consumptions are practically identical, being 238.4 and 238.2 cc., in rest and hyperventilation, respectively. The basal metabolic rate also fails to show any significant variation; the mean value in resting being − 8.4 per cent as compared to − 9.0 per cent in hyperventilation.

It thus appears that moderate hyperventilation *per se* in a series of five determinations on apparently healthy young males has no significant effect on the oxygen consumption or basal metabolic rate.

DISCUSSION

Standards for determining the basal metabolic rate as a deviation from normal are now well accepted when the DuBois standards (1) are used whereby body surface area is considered. Failure to appreciate this relationship tends to invalidate experiments performed prior to 1916. The method of indirect calorimetry using a closed circuit such as is commonly used for clinical determination of oxygen consumption was considered unreliable for our determinations because of the difficulty of interpreting a spirometer record when ventilation is excessive and because in that method a standard respiratory quotient is assumed.

There are many factors that affect the oxygen consumption and might lead to an error of interpretation of these results showing a markedly increased basal metabolic rate in diabetic acidosis. In the main, these are all of the well known factors that affect the basal metabolic rate. Fever will invariably increase the metabolic rate. There was

no appreciable elevation of body temperature in the group studied. There is doubt that these patients were truly basal, though in many respects they might be considered more basal than the patients usually tested, for all were lethargic or hypoactive, all had been fasting for more than 12 hours and most had been in bed for more than 24 hours. The control group was not apparently in a more basal condition than was the experimental group. It is conceivable that, with the hyperventilation of these patients in acidosis, the actual muscular work involved in hyperventilation might contribute to the increased oxygen consumption. It has been demonstrated (12) that only 3.7 per cent of the oxygen consumed is used to satisfy the extra needs of hyperventilation alone up to a respiratory exchange of 22 liters per minute. Our findings in Table II fail to show any significant change in oxygen consumption as a result of moderate hyperventilation. Hyperventilation, therefore, does not apparently significantly affect the magnitude of the changes of the basal metabolic rate reported here. Only two patients had a respiratory pattern that could be considered Kussmaul in type.

Practically every patient was severely dehydrated and weighed less than he would under normal conditions. Therefore, using his assumed normal weight would be more reliable than the actual measured weight when dehydrated. It was found that a variation of 10 pounds of weight did not affect the calculated basal metabolic rate appreciably. The weight used in calculating the basal metabolic rate in these studies was the weight as measured after the first day of therapy when the previous dehydration had been corrected to a great extent. Had the admission weight been used, the calculated metabolic rate would have been even higher. It has been noted (13) that sleep itself tends to lower the basal metabolic rate 10 to 13 per cent. Since most patients in this group were lethargic and some of them sleeping, this factor would tend to make these results more valid in that the error would have been a result too low rather than too high. Mental effort has been shown to increase the basal metabolic rate only negligibly, while strong emotion caused an increase of only 5 to 10 per cent (13).

Fulton (14) has noted that hyperventilation in itself affects the carbon dioxide : oxygen ratio in such a manner that the determined respiratory quotient in patients who are hyperventilating may vary with this factor of hyperventilation irrespective of any true metabolic change. In addition to the calculations tabulated, the basal metabolic rate was also calculated for each patient using an assumed respiratory quotient of 0.82. This served to raise the calculated basal metabolic rate in each case, so these figures were not used.

Thus every incidental factor which could reasonably have altered the metabolic rate in these patients appears incapable of explaining, in itself or in combination with others, the marked and statistically significant increase in oxygen consumption and metabolic rate which is found. The conclusion appears justified that diabetic acidosis is associated with a real increase in the oxygen consumption of the body as a whole. That this is not a uniform acceleration of metabolism involving all the tissues is indicated by previous work from this laboratory (15) which demonstrated a significant reduction in cerebral oxygen consumption in this condition. Diabetic acidosis is not the only state in which the oxygen consumption of the brain appears to behave independently of that of the body as a whole. Recently two groups working independently have found no cerebral counterpart to the increased total metabolism of hyperthyroidism (16, 17).

The significance of this increase in oxygen consumption in diabetic acidosis remains obscure. It may be of interest, however, to point out that this finding is compatible with the *in vitro* observations of Polis and associates (18) in this laboratory on a possible mechanism of insulin action. This group found insulin to be capable of increasing the efficiency of the synthesis of high energy phosphorous compounds with energy released by an oxidative process. If an important defect resulting from the relative insulin deficiency of diabetes were to reside in the conversion of oxidative energy to useful work through the energy-rich phosphate bond, then an acceleration of oxidative processes might be necessary to maintain normal cellular function in uncontrolled diabetes. This thesis is further supported by preliminary observations which have failed to demonstrate an increase in cellular uptake of P^{32} in the face of this increased rate of oxidation.

SUMMARY

1. Total oxygen consumption and metabolic rate was studied in 11 patients in diabetic acidosis and in seven non-diabetic controls.

2. There is a marked and statistically highly significant increase in the metabolic rate of the group in diabetic acidosis compared with expected normal values and with values found in the non-diabetic controls.

3. Hyperventilation *per se*, in a series of five determinations on apparently healthy young males showed no significant effect on the oxygen consumption or basal metabolic rate.

4. The possible significance of these findings is discussed.

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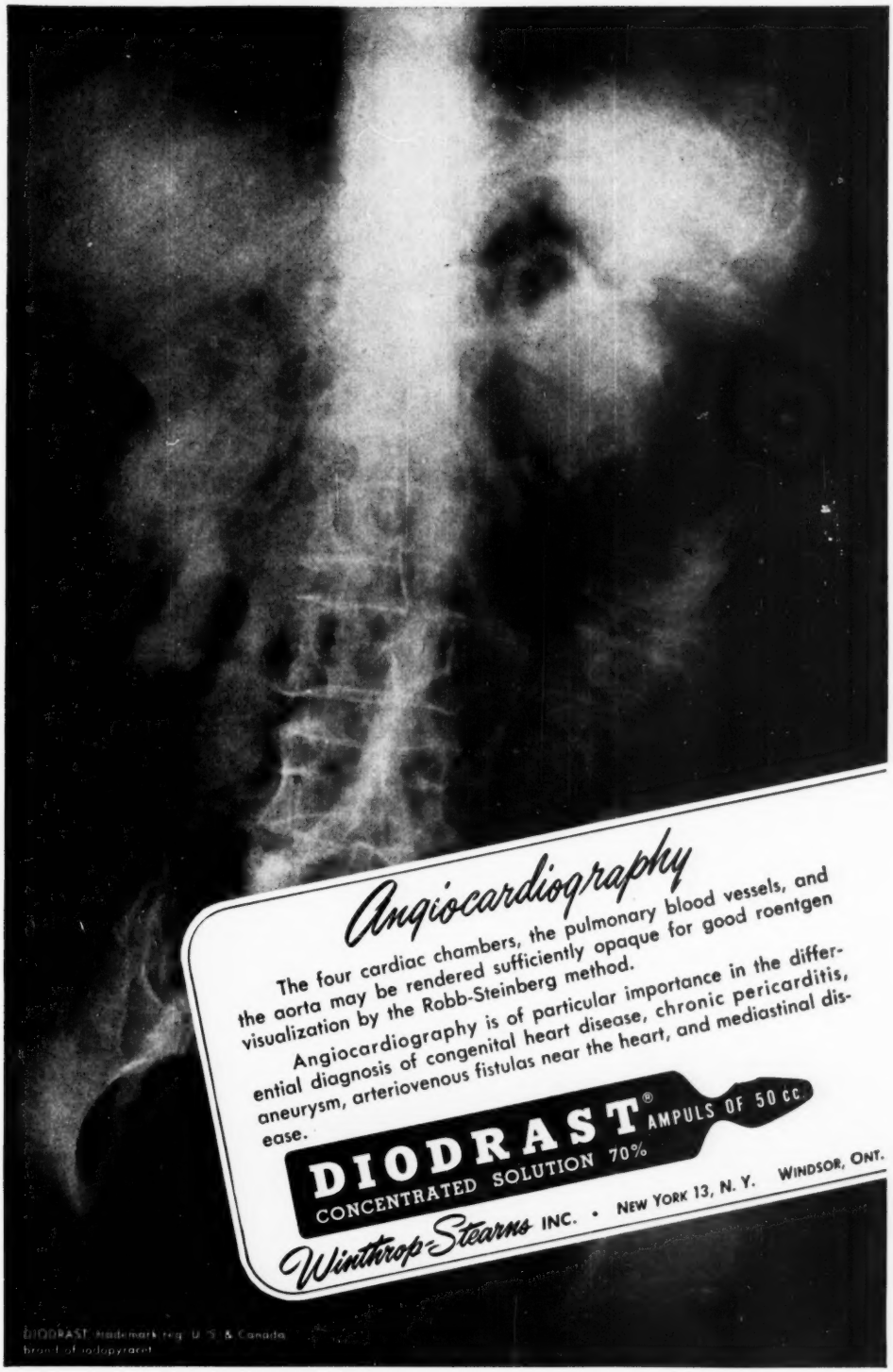
REFERENCES

1. DuBois, D., and DuBois, E. F., Clinical calorimetry. X. A formula to estimate the approximate surface area if height and weight be known. Arch. Int. Med., 1916, **17**, 863.
2. Allen, F. M., and DuBois, E. F., Clinical calorimetry. XVII. Metabolism and treatment in diabetes. Arch. Int. Med., 1916, **17**, 1010.
3. Wilder, R. M., Boothby, W. M., and Beeler, C., Studies of the metabolism of diabetes. J. Biol. Chem., 1922, **51**, 311.
4. Holten, C., The respiratory metabolism in diabetes and the influence of insulin upon it. J. Metabol. Research, 1924, **6**, 1.
5. Hedon, L., Le quotient respiratoire et le métabolisme de base dans le diabete pancréatique expérimental. Arch. Internat. de Physiol., 1927, **29**, 175.
6. Bernstein, S., and Falta, W., Über den einfluss der ernahrungsweise auf den ruhenuchternumsatz bie normalen diabetischen individuen. Deutsches Arch. f. klin. Med., 1916, **121**, 95.
7. Richardson, H. B., and Mason, E. H., Clinical calorimetry. XXXIII. The effect of fasting in diabetes as compared with a diet designed to replace the foodstuffs oxidized during a fast. J. Biol. Chem., 1923, **57**, 587.
8. Duncan, G. G., *in:* Diseases of Metabolism, edited by Duncan, G. G. W. B. Saunders Co., Philadelphia, 1947, 2nd edition, p. 18.
9. Joslin, E. P., Root, H. F., White, P., Marble, A., and Bailey, C. C., The Treatment of Diabetes Mellitus. Lea & Febiger, Philadelphia, 1946, p. 262.
10. DuBois, E. F., Basal Metabolism in Health and Disease. Lea & Febiger, Philadelphia, 1936, p. 277.
11. Macleod, J. J. R., Physiology in Modern Medicine, edited by Bard, P. C. V. Mosby Co., St. Louis, 1938, 8th edition, p. 731.
12. Muller, E. A., Michaelis, H. F., and Muller, A., Der energieaufwand fur die atmung beim memschen. Arbeitsphysiol., 1942, **12**, 192.
13. Best, C. H., and Taylor, N. B., Physiological Basis of Medical Practice. The Williams & Wilkins Co., Baltimore, 1945, 4th edition, p. 522.
14. Howell, W. H., A Textbook of Physiology, edited by Fulton, J. F. W. B. Saunders Co., Philadelphia, 1949, 16th edition, p. 1004.
15. Kety, S. S., Polis, B. D., Nadler, C. S., and Schmidt, C. F., The blood flow and oxygen consumption of the human brain in diabetic acidosis and coma. J. Clin. Invest., 1948, **27**, 500.
16. Sokoloff, L., Wechsler, R. L., Balls, K., and Kety, S. S., The relation of the cerebral O_2 consumption to the total body metabolism in hyperthyroidism. J. Clin. Invest., 1950, **29**, 847.
17. Scheinberg, P., Cerebral circulation and metabolism in hyperthyroidism. J. Clin. Invest., 1950, **29**, 1010.
18. Polis, B. D., Polis, E., Kerrigan, M., and Jedeikin, L., Effect of insulin and adenosinetriphosphatase on a reaction coupling oxidation with phosphorylation. Arch. Biochem., 1949, **23**, 505.



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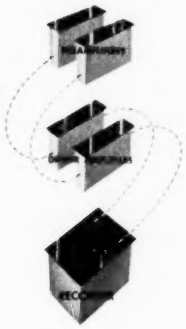

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